Regulation of lipid metabolism in macrophages by long-chain metabolites of α-tocopherol

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Table of contents

I. List of tables VIII

II. List of figures IX

III. List of abbreviations X

1 The vitamin theory 12

2 Vitamin E 13

2.1 Milestones in vitamin E research 13

2.2 Recommendations for vitamin E intake 15

2.2.1 Vitamin E supply 16

2.2.2 Vitamin E status 16

2.2.3 Toxicology 17

2.3 Digestion of vitamin E 18

2.3.1 Bioavailability of vitamin E 18

2.3.2 Intestinal absorption and distribution of vitamin E 18

2.3.3 Storage of vitamin E 19

2.4 Hepatic metabolism of vitamin E 19

2.4.1 Principles of vitamin E metabolisation 19

2.4.2 Metabolisation efficiency 21

2.4.3 Verification of systemic LCM availability 21

3 Long-chain metabolites of vitamin E 22

3.1 Synthesis of vitamin E long-chain metabolites 22

3.2 Analysis of vitamin E long-chain metabolites 23

3.3 Biological activities of vitamin E long-chain metabolites 24

3.3.1 Anti-inflammatory activities 25

3.3.2 Anti-carcinogenic effects 25

3.3.3 Effects on lipid homeostasis 26

3.3.4 Other known biological effects 27

3.3.4.1 Interaction with pharmaceuticals 27

3.3.4.2 Regulation of LCM formation 27

3.3.4.3 Structure specific effects 27

4 Atherosclerosis 27

4.1 The relevance of the forgotten lipids for atherosclerosis 28

4.2 Mechanisms of TRL mediated development of atherosclerosis 29
5 Aim of the thesis 31
6 Manuscript overview 32
7 Manuscripts 43
7.1 Manuscript I 43
7.2 Manuscript II 60
7.3 Manuscript III 69
7.4 Manuscript IV 78
7.5 Manuscript V 88
7.6 Manuscript VI 99
7.7 Manuscript VII 146
7.8 Manuscript VIII 165
7.9 Manuscript IX 194
7.10 Manuscript X 213
8 Discussion 246
8.1 Contribution of the integrated manuscripts to the progress of research in the field of vitamin E-derived LCMs 246
8.1.1 Biological functions of the LCMs 247
8.1.1.1 Modulation of lipid metabolism in macrophages 247
8.1.1.2 Modulation of the inflammatory processes 249
8.1.2 Affected signaling pathways 250
8.1.3 Functional comparison of metabolites and vitamin precursor 251
8.2 Limitations of current LCM research 252
8.2.1 Utilized experimental models 252
8.2.1.1 In vitro models 253
8.2.1.2 In vivo models 254
8.2.2 Dosage and uptake of the LCMs 256
8.2.3 Utilized compounds 258
8.2.4 Modes of action 260
8.2.4.1 The mitogen-activated protein kinase pathway 261
8.2.4.2 The nuclear factor 'kappa-light-chain-enhancer' of activated B-cells pathway 261
8.2.4.3 Ion channel mediated signaling pathways 262

8.2.4.4 Peroxisome proliferator-activated receptor mediated signaling .. 262
8.2.4.5 Pregnane-X-receptor mediated signaling 263
8.3 Future perspectives of LCM research 263
8.3.1 *In vitro* studies 264
8.3.2 *In vivo* studies based on animal models 264
8.3.2.1 Investigation of hepatic metabolism 265
8.3.2.2 Tissue distribution 265
8.3.2.3 Pharmacokinetics 265
8.3.2.4 LCM stability 265
8.3.2.5 Identification of physiological relevant forms of vitamin E LCM ... 266
8.3.3 Human studies 266
9 Summary 268
10 Zusammenfassung 270
A References ..... A
B Eigenständigkeitserklärung ..... Q
C Publications ..... R
D Danksagung ..... T
E Supplement V

I. List of tables

Table 1: Recommended daily intake of vitamin E by countries and organizations worldwide. 16

Table 2: Concept of a dual distribution of vitamin E roles in human health. 17

Table 3: Subset of analyzable LCMs in human blood with their corresponding preparation and detection methods. 24

II. List of figures

Figure 1: The different forms of TOHs and TEs. 13

Figure 2: Milestones of vitamin E and LCM research. 14

Figure 3: The hepatic metabolism of vitamin E. 20

Figure 4: Semisynthesis of α-T-13'- and δ-T-13'-LCMs of vitamin E from garcinoic acid (δ-TE-13'-COOH). 22

Figure 5: The LPL pathway and its potential involvement in foam cell formation and atherosclerosis. 30

Figure 6: Schematic overview about the manuscripts included the thesis. 32

Figure 7: Personal contribution to the progress of research in the field of vitamin E LCMs. 247

Figure 8: Schematic overview about major limitations of current LCM research. 252

Figure 9: Schematic overview on major aims of future LCM research. 263

III. List of abbreviations

5-LO	5-lipoxygenase
13’-COOH	13’-carboxychromanol
13’-OH	13’-hydroxychromanol
α-TTP	α-tocopherol transfer protein
α-T-13’-COOH	α-tocopherol-derived 13’-carboxychromanol
ABCA1	ATP-binding cassette transporter A1
Apo	Apoprotein
ANGPTL	Angiopoietin-like
CEHC	Carboxy-ethyl-hydroxychromanol
CD36	Cluster of differentiation 36
CoA	Coenzyme A
COX	Cyclooxygenase
CYP	Cytochrome P450
DGE	German Society of Nutrition
ERK	Extracellular signal-regulated kinase
FFA	Free-fatty acid
GA	Garcinoic acid
GC	Gas chromatography
ICM	Intermediate-chain metabolite
IL	Interleukin
IκB	Inhibitor of κB
iNOS	Inducible nitric oxide synthase
JNK	C-Jun N-terminal kinase
LDL	Low-density lipoprotein
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
HPLC	High-performance liquid chromatography
LCM	Long-chain metabolite

LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
oxLDL	Oxidized low-density lipoproteins
p38	Mitogen-activated protein kinase p38
PBMC	Peripheral Blood Mononuclear Cells
PG	Prostaglandin
P-gp	P-glycoprotein
PLIN2	Perilipin 2 (adipophilin)
PMNL	Polymorphonuclear leukocytes
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
Ras	Rat sarcoma
RDD	Recommended daily dose
RP	Research priority
RXR	Retinoid X receptor
SCM	Short-chain metabolite
TE	Tocotrienol
TOH	Tocopherol
TEQ	Tocopherol equivalents
TRL	Triglyceride-rich lipoproteins
TX	Thromboxane
VLDL	Very low-density lipoproteins

1 The vitamin theory

The theory on the existence of nutritional factors essential for human health accompanied human history over centuries. Already the ancient Egyptians recognized that night blindness – a disease caused by vitamin A deficiency – could be treated by the administration of liver (Al Binali 2014). Scurvy, a disease that became most prevalent in sailors between the 15th and 18th century, represents another prominent example for a vitamin deficiency disease. The Scottish naval surgeon James Lind (1716 - 1794) recognized that scurvy could be treated by the consumption of citrus fruits. Nevertheless, it took additional 200 years till the identification of vitamin C (Souganidis 2012).

In 1912, the dietetic factors essential for the treatment of 'deficiency disorders', *i.e.* human beriberi, scurvy, rickets and pellagra, were entitled as 'vitamin' by the polish scientist Casimir Funk (1884 – 1967). The term 'vitamin' was a combination of 'vita' = life and 'amine' = a nitrogenous substance essential for life (Piro et al. 2010). The American scientist Elmer McCollum (1879-1967) criticized this nomenclature, since the 'fat-soluble A' – a substance he had recently discovered – did not contain nitrogen. To overcome these discrepancy, the British biochemist Sir Jack Cecil Drummond (1891 – 1952) suggested to complement the term 'vitamin' with the letters of the alphabet (Rosenfeld 1997).

Vitamin research of the early nineteenth century was characterized by slow and stepwise progression. Especially animal models enabled the advantage of nutritional research, since deficiency experiments generated new insights into the significance of nutrition for health. To name a few: Christiaan Eijkman (1858 – 1930) observed that chickens fed with polished rice developed polyneuritis, *i.e.* the equivalent to human beriberi. Eijkman concluded that the starch in polished rice carries a toxin that is normally neutralized by the rice bran. Gerrit Grijns (1865 – 1944) continued the investigations of Eijkman. He hypothesized that beriberi is caused by the lack of a vital nutritional component in the chickens diet, which was later identified as vitamin B_1 (*i.e.* thiamin) (Semba 2012). Wilhelm Stepp (1882 – 1964) found that growth of young mice was promoted when he provided them a dough made of flour and milk. After he extracted the dough with a mixture of alcohol and ether, the mice were only able to survive up to three weeks. Interestingly, when the extracted substances were added back to the dough, the survival rate of the mice was normalized. Stepp concluded that milk contains a substance essential for the survival of mice (vitamin A) (Semba 2012). Although animal studies had a significant impact on the progression of vitamin research, it was the isolation and chemical synthesis of the vitamins that enabled their detailed characterization. The chemical synthesis of vitamins paved the way for modern vitamin research, including the development of dietary guidelines, the discovery of vitamin deficiency disorders, the fortification of vitamins in foods and the production of vitamin supplements. As an expression of the importance of vitamin research for human health, the work of numerous scientist on the field was awarded with Nobel Prizes in Chemistry, Physiology or Medicine (Souganidis 2012).

2 Vitamin E

The term vitamin E summarizes a group of lipophilic molecules, which can be classified as tocopherols (TOHs), tocotrienols (TEs) and vitamin E related structures. All vitamin E molecules consist of a chromanol ring and a covalently connected phytyl-like side chain. Based on the saturation of the side chain, the vitamin E molecules are divided into TOHs (bearing a saturated side chain) and TEs (carrying an unsaturated side chain). In addition, the methylation pattern of the chromanol ring determines the classification as α-, β-, γ- or δ-form of the respective TOHs or TEs (Figure 1). Natural forms of vitamin E exist in the *RRR*-configuration (TOH) or the *R*-configuration (TE), whereas synthetic vitamin E is a mixture of the different stereoisomers (extensively reviewed in manuscript VI (Kluge et al. 2016)). Interestingly, due to its ability to preserve fertility in rats as well as preventing ataxia with vitamin E deficiency (AVED) in humans, α-TOH is the only form of vitamin E with proven vitamin property (Azzi 2019).

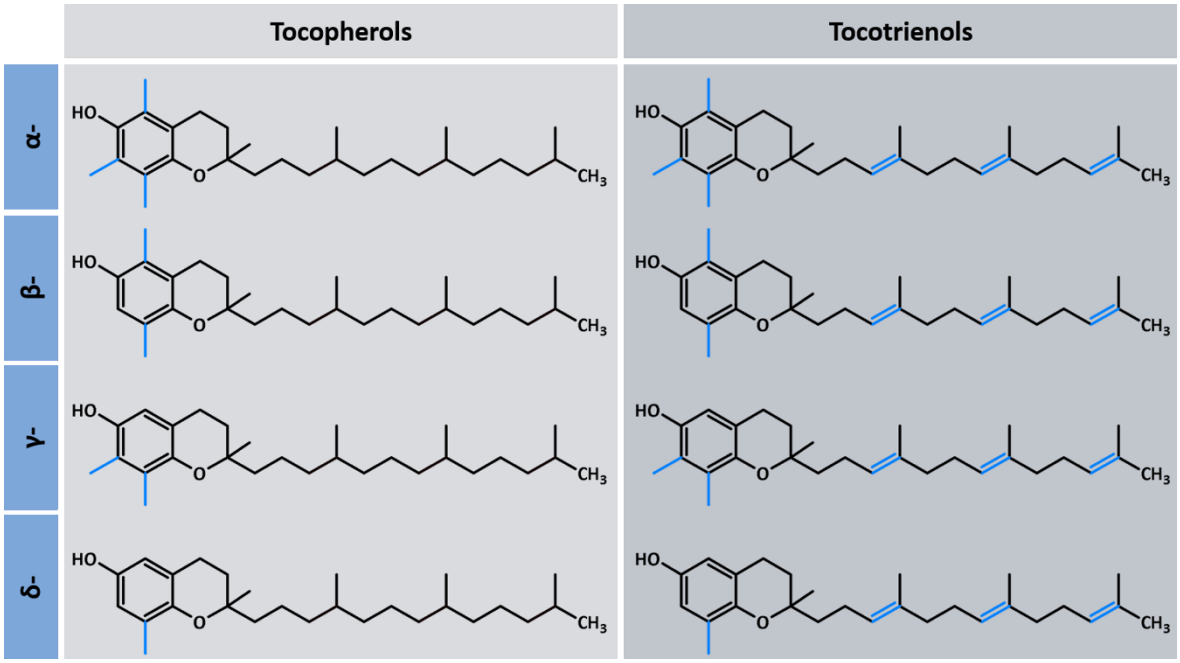

Figure 1: The different forms of TOHs and TEs. Vitamin E related structures are not shown. Adapted from (Kluge et al. 2016).

2.1 Milestones in vitamin E research

In a recently published article, Birringer *et al.* presented their personal selection of key findings in vitamin E history (Birringer et al. 2019). The following section will provide a modified version of this selection, which is predominantly focused on milestones of the current research on vitamin E long-chain metabolites (LCMs) as well as on previous findings that enabled their discovery (**Figure 2**).

The discovery of vitamin E (1922)

In 1922, Katherine J. Scott Bishop and Herbert M. Evans observed that rats fed with a diet containing casein, cornstarch and lard supplemented with butterfat and salt are not capable of reproduction (Evans und Bishop 1922). However, the ability for reproduction was restored after the rats received fresh green leaves of lettuce. Based on this observation, Evans and Bishop concluded that natural food contains a 'factor X' that prevents sterility (Evans und Bishop 1922). After the description of its chemical and biological activities, the unknown compound was named 'α-tocopherol' ('tokos' = childbirth and 'phero' = to bear) (Evans et al. 1936). The ability to prevent free radical induced lipid oxidation made α-TOH the most important lipid 'chain-breaking' antioxidant (Weber et al. 2019). The first characterization of the α-TOH structure was presented in 1938 by the German chemist Erhard Fernholz (Fernholz 1938). In the same year Paul Karrer and Otto Isler accomplished the first chemical synthesis of *DL* α-TOH (Karrer et al. 1938).

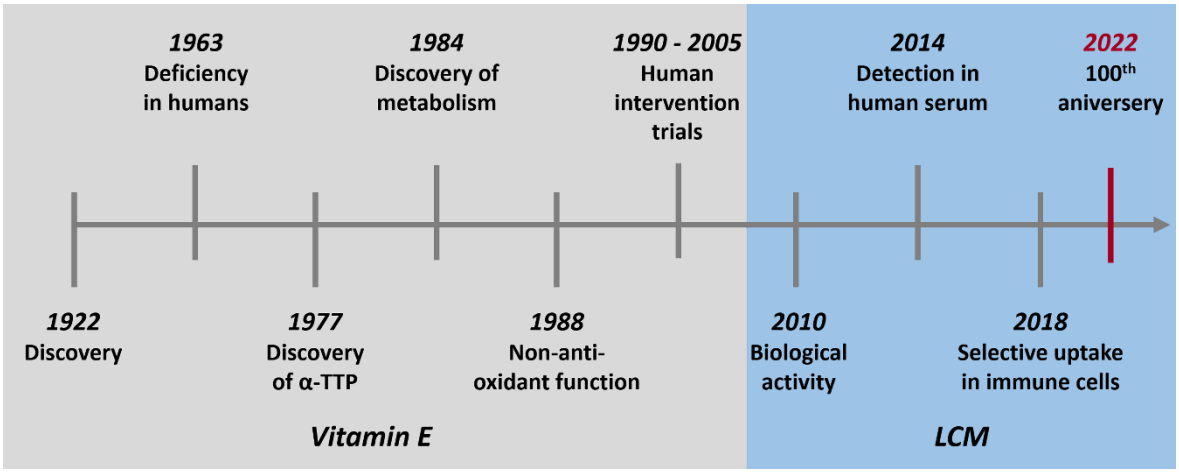

Figure 2: Milestones of vitamin E and LCM research. Adapted from (Birringer et al. 2019). LCM (long-chain metabolite).

Discovery of vitamin E metabolism (~ 1984)

In 1984, Chiku *et al.* unrevealed the metabolism of vitamin E in a rat model. After the administration of deuterated δ-TOH, the scientist were able to detect the water-soluble short-chain metabolite (SCM) δ-carboxy-ethyl-hydroxychromanol (CEHC) – the end product of vitamin E metabolism – in urine of the rats (Chiku et al. 1984). Beside δ-CEHC, the occurrence of structurally related α- and γ-CEHC was also described. The discovery of vitamin E metabolism was a key finding for later LCM research, since it in principle promoted the investigation on vitamin E metabolites.

Discovery of non-anti-oxidative functions of vitamin E (~ 1988)

Azzi and colleagues were the first to describe a non-anti-oxidative function of vitamin E. They showed that protein kinase C activity could be inhibited by α- or β-TOH (Mahoney und Azzi 1988). In addition, further specific non-anti-oxidative functions, such as inhibition of platelet adhesion and aggregation, inhibition of 5-lipoxygenase (5-LO) activity and modulation of α-

tocopherol transfer protein (α-TTP) expression, have been described for *RRR*-α-TOH (Znigg und Azzi 2004). In the 2000s, an additional gene regulatory potential was discovered for TOHs and TEs (Landes et al. 2003; Landrier et al. 2009; Valastyan et al. 2008).

Biological function and semi-synthesis of vitamin E long-chain metabolites (2010)

In 2010, an investigation of Birringer *et al.* provided two milestones in the field of LCM research: (i) It was shown that vitamin E-derived LCMs reveal pro-apoptotic and anti-proliferative activities in the human liver cancer cell line HepG2. Interestingly, the respective vitamin precursors showed no effect on cell viability or proliferation (Birringer et al. 2010). (ii) Birringer *et al.* provided a simplified route for the semi-synthesis of α- and δ-LCMs from the natural compound δ-tocotrienolic acid (δ-TE-13'-COOH), better known as garcinoic acid (GA). The study of Birringer *et al.* was the initial description of a biological activity of the vitamin E-derived LCMs and the study provided first hints that the LCMs could probably represent the biologically active forms of TOHs and TEs. In addition, the described semi-synthetic route enabled the production of large amounts of α- and δ-LCMs in high purity, which had a major impact on future LCM research (Birringer et al. 2010).

Evidence for a systemic relevance of vitamin E long-chain metabolites (2014)

In 2014, Wallert *et al.* were the first to detect α-tocopherol-derived 13'-carboxychromanol (α-T-13'-COOH) in the serum of a healthy, middle-aged, non-smoking male, who received a balanced diet with no additional supplementation of vitamin E (Wallert et al. 2014b). Hence, Wallert and coworkers hypothesized that the LCMs are likely formed during the hepatic metabolism of vitamin E. Afterwards, the newly formed metabolites are transferred into blood circulation, which enables their transport to various sites of action. This was a key finding in the field, since it provided evidence for a systemic relevance of the LCMs, at least for the α-forms. The occurrence of α-T-13'-COOH in human blood was later confirmed by others (Torquato et al. 2016b; Pein et al. 2018).

Selective uptake of α-T-13'-COOH into immune cells (2018)

The concept that vitamin E-derived LCMs are transferred to peripheral tissues of the human body to convey regulatory effects, raised a central question: Are the metabolites absorbed by peripheral cells and tissues? In 2018, Pein *et al.* were the first to show the selective uptake of α-T-13'-COOH by murine peritoneal leukocytes. In addition, their investigation also provided a description of uptake kinetics for vitamin E LCMs (Pein et al. 2018), which represented a great limitation of previous *in vitro* studies on that topic.

2.2 Recommendations for vitamin E intake

The recommendations for the dietary intake of vitamin E vary between different nutritional authorities, depending on the biomarkers chosen to set their reference values. To overcome these differences, a recent publication suggested to consistently base the intake recommendations for vitamin E on its anti-oxidative activity in lipophilic compartments

(Raederstorff et al. 2019). In line with this concept, the reference values of the German Society of Nutrition (DGE) are based on the capacity of the respective vitamin E form to prevent lipid peroxidation. In the D-A-CH area (Germany, Austria, Switzerland), a daily vitamin E intake of 12 - 15 α-tocopherol equivalents (TEQ) is recommended for adults (DGE 2019). The calculation of a TEQ is based on the determination of the biological activity of the respective TOHs and TEs in a fetus absorption test in rats (Kamal-Eldin und Appelqvist 1996). For example, 1 mg α-TOH corresponds to one TEQ, while 1 mg γ-TOH only corresponds to 0.1 TEQ. In contrast, the recommended daily dose (RDD) of vitamin E in the USA (15 mg/d) is based on the amount of vitamin E that was necessary to avoid peroxide-induced hemolysis of blood samples from vitamin E deficient people (Péter et al. 2019). However, the use of this biomarker for the calculation of the daily vitamin E supply is controversially discussed (Azzi 2018). An overview about the recommendations of different national and international authorities is provided in **Table 1.**

Table 1: Recommended daily intake of vitamin E by countries and organizations worldwide. Adapted from (Péter et al. 2019). *D-A-CH* (Germany-Austria-Switzerland), *EFSA* (European Food Safety Authority), *IOM* (US Institute of Medicine of the National Academy of Science), *NCM* (Nordic Council of Ministers), *WHO/FAO* (World Health Organization/Food and Agriculture Organization of the United Nations).

	D-A-CH (2015)	EFSA (2015)	NCM (2014)	WHO/FAO (2004)	IOM (2004)
Age (years)	≥ 19	≥ 10	≥ 18	≥ 19	≥ 19 - 50
Men (mg/day)	12 - 15	13	10	10	15
Women (mg/day)	11 -12	11	8	7.5	15

2.2.1 Vitamin E supply

In a recent meta-analysis on global vitamin E supply, a median intake of 6.2 mg/d α-TOH and 1 mg/d γ-TOH was calculated using data of 249 637 participants from 46 countries. Based on a recommended daily intake of 15 mg α-TOH (for adults), 82% of the global population was insufficiently supplied with vitamin E (Péter et al. 2019). Interestingly, the level of vitamin E supply was highly dependent on the geographical region. While 91% of the American population were below the RDD of 15 mg α-TOH, only 55% of the European population revealed an insufficient α-TOH supply (Péter et al. 2019). In Germany, the vitamin E supply of about 50% of the adult population was below the DGE recommendation of 12 - 15 TE/d (MRI 2008). However, vitamin E undersupply had no influence on the general health status. Therefore, scientist are currently discussing an adjustment of the reference values (Péter et al. 2019).

2.2.2 Vitamin E status

The US Food and Nutrition Board defined a α-TOH plasma concentration of 12 µmol/l as a cut-off value for vitamin E deficiency in healthy adults, since plasma levels below 12 µmol/l were

associated with miscarriage and increased erythrocyte fragility (IOM 2000; Shamim et al. 2015). In addition, plasma α-TOH levels of < 8 µmol/l led to the development of ataxia, peripheral neuropathy or skeletal myopathy (Péter et al. 2019). It was also shown that low plasma α-TOH concentrations are linked to an increased risk for mild cognitive impairments and Alzheimer's disease (Mangialasche et al. 2012). In contrast to the negative effects of vitamin E deficiency, different epidemiological studies provided evidence that α-TOH concentrations of ≥ 30 µmol/l are associated with health benefits (Péter et al. 2019). This concept is strengthened by the finding that urinary excretion of α-CEHC increases at a α-TOH serum threshold of 30 µmol/l, which is suggested as an indicator for sufficient vitamin E supply (Lebold et al., 2012). Unfortunately, the unadjusted worldwide median for circulating α-TOH was determined as only 22.1 µmol/l (Péter et al. 2019). Interestingly, plasma vitamin E concentrations above 45 µmol/l – resulting from pharmacological interventions – were associated with therapeutic benefits on immune function, cardiovascular health, liver health and cognitive function (**Table 2**) (Péter et al. 2019).

Table 2: Concept of a dual distribution of vitamin E roles in human health. Adapted from (Péter et al. 2019). *CV* (cardiovascular)

Vitamin E	**Essentiality**		**Health benefits**	
Status	Deficient	Suboptimal	Desirable	Therapeutic
Serum concentration	≤ 12 µmol/l	13 - 29 µmol/l	30 - 44 µmol/l	≥ 45 µmol/l
Intake	< 15 IU/day	15 - 54 IU/day	55 - 249 IU/day	≥ 250 IU/day
Health impact	Hemolysis neurological symptoms, miscarriage	Absence of deficiency normal erythrocyte stability	Decreased risk for noncommunicable diseases	Immune function CV health liver health cognitive function

2.2.3 Toxicology

The intake of vitamin E from different food sources is generally considered safe. However, different national and international authorities have established a vitamin E intake of 300 – 1000 mg/d as the upper tolerable intake level (EFSA, Panel on Dietetic Products, Nutrition and Allergies 2015; US National Research Council und US Institute of Medicine 2000). In line with this, the administration of a single dose of 300 mg mixed TEs did not led to any adverse effects in healthy volunteers (Yap et al. 2001). Nevertheless, the potential toxicity of vitamin E has always been an issue in the scientific community. For example, animal studies revealed that persistent high-dose vitamin E supplementation interfered with blood clotting and was therefore associated with an increased risk of hemorrhagic stroke (Zondlo Fiume 2002). Further, a meta-analysis from 2005 provided evidence that vitamin E supplementation with at

least 400 IU/d increases cancer risk and all-cause mortality, which had a great impact on the discussion about the toxicity of vitamin E (Miller et al. 2005). However, these results have been disproved by a recent meta-analysis. Here, the analysis of 250 000 participants with a vitamin E supplementation between 16.5 and 5000 IU/d showed no clear evidence that vitamin E increases all-cause mortality in humans (Köpcke 2019). Therefore, vitamin E intake seems to be safe even at much higher doses than the current RDD of 15 mg α-TOH per day.

2.3 Digestion of vitamin E

Based on its lipophilic character, dietary vitamin E is primarily ingested from lipid-rich foods. Especially plant-based oils, such as wheat germ oil (151 - 192 mg α-TOH/100 g), sunflower oil (33 - 59 mg α-TOH/100 g) or soya oil (61 - 70 mg γ-TOH/100 g) contain relatively high amounts of vitamin E (Shahidi und Camargo 2016).

2.3.1 Bioavailability of vitamin E

The bioavailability of vitamin E depends on different factors that have been summarized by Borel *et al.* with the acronym 'SLAMENGHI'. Each letter stands for one factor: (S) species of vitamin E, (L) linkage (*e.g.* esterification of vitamin E), (A) amount of vitamin E in the meal, (M) matrix in which vitamin E is incorporated, (E) effectors of absorption and bioconversion (*e.g.* lipids, fiber, drugs), (N) Nutrient (*i.e.* vitamin E) status of the host, (G) genetic factors, (H) host-related factors (*e.g.* sex, age), (I) mathematical interactions (*e.g.* to differentiate between single and combination effects) (Borel et al. 2013). The concept of Borel *et al.* is strengthened by findings from other studies: (i) Unconjugated natural *RRR*-α-TOH has the highest absorption rate of all vitamin E forms (Nagy et al. 2013). (ii) The bioavailability of vitamin E increases due to the parallel ingestion of lipids (Bruno et al. 2006). (iii) The bioavailability of vitamin E varies between different food matrices (Jeanes et al. 2004). In addition, different human studies observed an absorption rate between 55 and 81% of the administrated α-TOH (Mac Mahon und Neale 1970; Novotny et al. 2012). The peak concentration of α-TOH in plasma was reached 5 h and 9 h post-administration.(Mac Mahon und Neale 1970).

2.3.2 Intestinal absorption and distribution of vitamin E

The intestinal absorption of vitamin E follows that of other lipophilic molecules (Rigotti 2007). Initially, the consumed vitamin E is released from its food matrix due to enzymatic processing by gastric lipases in the stomach (Borel et al. 2013). Further digestion of vitamin E appears in the intestinal lumen, where, among others, vitamin E esters from supplements, such as tocopherol acetate, are hydrolyzed to a non-esterified form. The emulsification of vitamin E and other lipophilic molecules is accomplished by bile acids, *i.e.* free vitamin E is incorporated into micelles formed with phospholipids and bile acids (Flory et al. 2019). Afterwards, the micelles are taken up by enterocytes via passive diffusion or receptor-mediated transport. The scavenger receptor class B type 1, the cluster of differentiation 36 (CD36) or the Niemann-Pick C1-like protein 1 were identified as receptors involved in the intestinal uptake of vitamin E (Reboul 2017; Goncalves et al. 2014; Yamanashi et al. 2017). Afterwards, vitamin E is

integrated into chylomicrons and secreted into the lymphatic system (Hacquebard und Carpentier 2005). Vitamin E secretion from enterocytes requires active transport via ATP-binding cassette transporter A1 (ABCA1) (Schmölz et al. 2016). After entering the circulation, chylomicrons are degraded by lipoprotein lipase (LPL), leading to the formation of chylomicron remnants (Hacquebard und Carpentier 2005). Vitamin E is not affected by LPL mediated hydrolysis of triglycerides and remains in the lipoprotein particle for further transport to the liver (Julve et al. 2016). Here, the chylomicron remnants are taken up by the hepatic low-density lipoprotein receptor-related protein 1 (Basford et al. 2011). The different forms of vitamin E are likely discriminated by α-TTP that promotes the incorporation of 2*R*- or *RRR*-α-TOH into very low-density lipoproteins (VLDL), while other vitamin E forms are either metabolized to their respective CEHCs for urinary excretion or secreted into bile. After its release from the liver due to the activity of ABCA1, α-TOH is incorporated into VLDL particles and transferred to extrahepatic tissues (Flory et al. 2019).

2.3.3 Storage of vitamin E

Due to its lipophilic character, vitamin E can be stored in different depots of the human body. A study in rats revealed that 90% of the overall α-TOH is stored in skeletal muscle (42%), the liver (28%) or in adipose tissue (21%) (Uchida et al. 2011; Bjørneboe et al. 1990). In general, the liver seems to be responsible for short-term storage, while the adipose tissue enables long-term storage of vitamin E (Machlin und Gabriel 1982). *In vitro*, vitamin E is predominantly located in cell membranes or cellular lipid-rich factions as well as in cytosolic lipid droplets (Saito et al. 2004; Traber und Kayden 1987). Interestingly, the intracellular TOH amount depends on the lipid content of the respective cell type (Saito et al. 2004).

2.4 Hepatic metabolism of vitamin E

The concept of vitamin E catabolism in the human body was already proposed in 1956 due to the detection of tocopheronic acid and tocopheronolactone, better known as Simon products, in human urine (Simon et al. 1956). The formation of Simon products was thought to occur because of an opening of the chromanol ring after a radicalic attack, leading to the formation of tocopherol quinone that is further degraded to tocopheronic acid and tocopheronelactone (Schmölz et al. 2016). However, this concept was disproved due to the discovery of δ-CEHC – a degradation product of δ-TOH with an intact chromanol ring and a shortened side chain – in human urine (Chiku et al. 1984). Interestingly, the Simon products were later identified as artifacts of the sample preparation process (Znigg 2007).

2.4.1 Principles of vitamin E metabolisation

This section will only provide a short overview about the key reactions of vitamin E degradation (**Figure 3**). For a detailed description of the vitamin E metabolism, the reader is referred to the manuscripts VI and VII. The metabolism of vitamin E in humans is predominantly localized in the liver. Interestingly, the metabolism can be increased in the presence of higher vitamin E levels to prevent toxic accumulation.

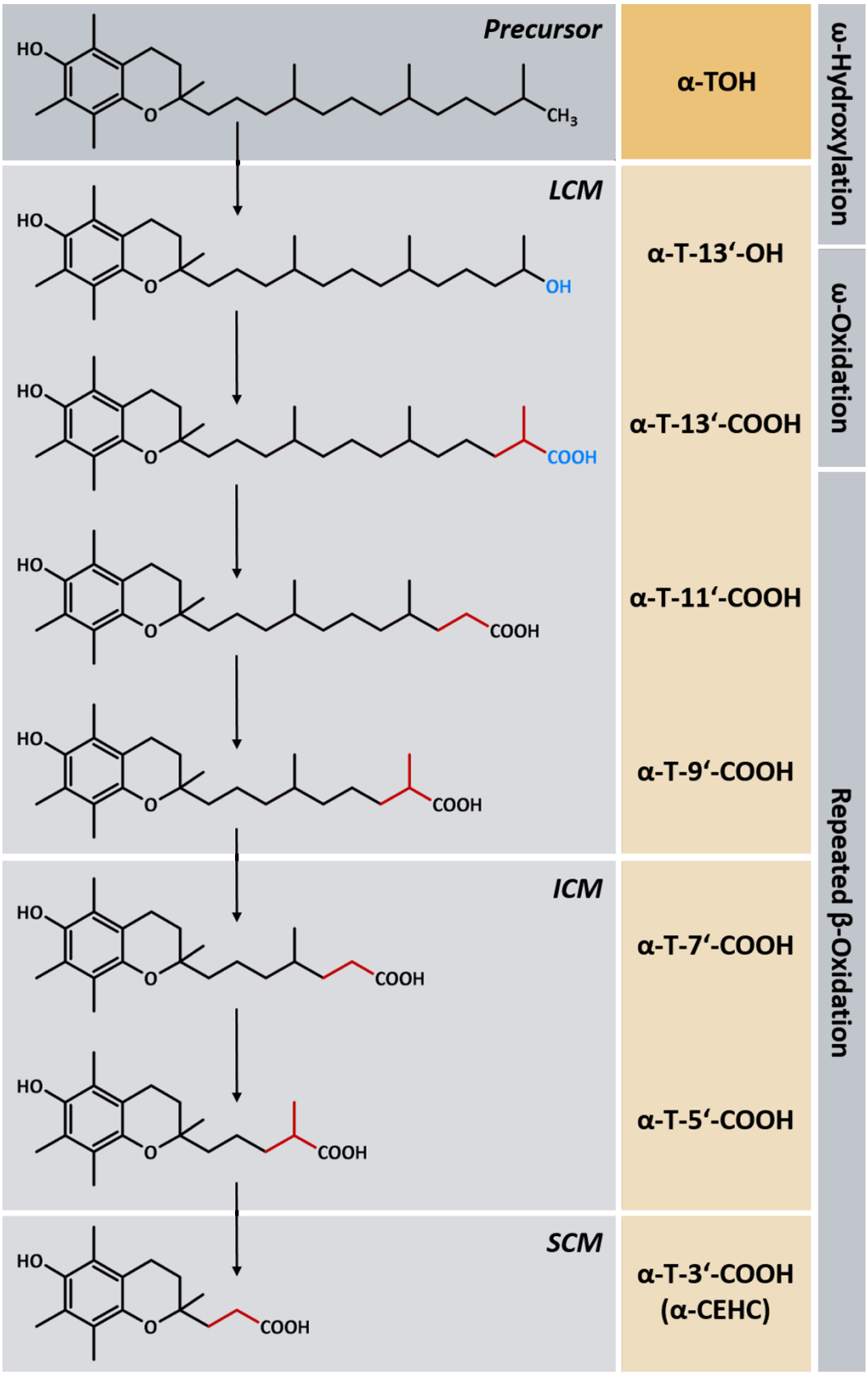

Figure 3: The hepatic metabolism of vitamin E. Adapted from (Schmölz et al. 2018). *CEHC* (carboxy-ethyl-hydroxychromanol), *ICM* (intermediate-chain metabolites), *LCM* (long-chain metabolites), *SCM* (short-chain metabolites).

The hepatic metabolism takes place in three different compartments: (i) endoplasmic reticulum, (ii) peroxisomes and (iii) mitochondria (Schubert et al. 2018).In general, the degradation of all TOH and TE forms follows the same metabolic route, which has been confirmed by the detection of α-, β-, γ- and δ-CEHC in urine (Chiku et al. 1984; Schultz et al. 1995; Swanson et al. 1999). Further, the classification of CEHCs as the metabolic endproducts of vitamin E catabolism indicates that only the side chain is modified during this process while the chromanol ring stays intact. In addition, the metabolisation of TEs require further enzymes, such as 2,4 dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase that are likely necessary for the degradation of the unsaturated side chain (Birringer et al. 2002). The initial and rate-limiting step of vitamin E metabolism is the formation of 13'-hydroxychromanol (13'-OH) metabolites via ω-hydroxylation of the terminal side chain by cytochrome P450 (CYP) 4F2 or CYP3A4 (Sontag und Parker 2002; Parker et al. 2000). Next, the terminal side chain of 13'-OH undergoes α-oxidation in the peroxisomes, resulting in the formation of 13'-carboxychromanol (13'-COOH). This reaction is probably mediated by alcohol- or aldehyde dehydrogenases, because an aldehyde intermediate is formed during the oxidation process. The mechanism for the further degradation of 13'-COOH is comparable to the degradation of branched-chain fatty acids. Hence, the terminal side chain is shortened by a stepwise removal of two carbon units via multiple β-oxidation cycles (Schubert et al. 2018). Overall, five β-oxidation cycles are necessary until the formation of the water-soluble end-product 3'-COOH, better known as CEHC (Birringer et al. 2001). The water-soluble SCMs are excreted via urine, while the LCMs and intermediate-chain metabolites (ICMs) are secreted into bile (Zhao et al. 2010). Because of enzymatic modifications during the hepatic metabolism, most of the urinary SCMs can be detected as sulfated- or glucuronidated conjugates (Birringer et al. 2001). In contrast, the lipophilic LCMs are predominantly excreted in their unconjugated form (Zhao et al. 2010).

2.4.2 Metabolisation efficiency

The metabolisation efficiency of the different vitamin E forms depends on at least three different factors: (i) the substitution of the chromanol ring, (ii) the saturation of the side chain and (iii) the source from which the vitamin is obtained (natural or synthetic) (Schmölz et al. 2016). The degradation rate of the vitamin E isoforms is decreased with an increasing number of methyl groups substituted to the chromanol ring (δ > γ/β > α-TOH/TE). Indeed, human trials revealed that round about 50% of the ingested γ-TOH are metabolized to γ-CEHC and excreted via urine, while only 1 - 3% of the consumed α-TOH were degraded to α-CEHC (Swanson et al. 1999; Schuelke et al. 2000). Further, the degradation of TOHs is less efficient compared with TEs. Interestingly, synthetic vitamin E forms revealed the highest and natural *RRR*-α-TOH the lowest metabolic rate (Sontag und Parker 2007).

2.4.3 Verification of systemic LCM availability

The discovery of vitamin E metabolism in humans together with the emerging evidence for important biological function of vitamin E LCMs, led to the hypothesis that the hepatic metabolism converts TOHs and TEs to their active forms (already described for the vitamins A

and D (Schubert et al. 2018)). In 2014, Wallert and coworkers detected nanomolar levels of α-T-13'-COOH in human serum, which was later confirmed by others (Wallert et al. 2014b; Torquato et al. 2016b; Giusepponi et al. 2017; Pein et al. 2018). The study showed for the first time that vitamin E LCMs are transferred into the blood circulation, providing strong evidence for their systemic relevance in the human body.

3 Long-chain metabolites of vitamin E

All TOH- and TE-derived metabolites with a side chain length between 13 and 9 carbon units are entitled as LCMs. Within these molecules, 13'-OHs and 13'-COOHs metabolites have emerged as promising bioactive molecules with a possible relevance for the regulation of physiologic functions. The growing interest on this new aspect of vitamin E research led to a significant increase of knowledge about the LCMs. This includes approaches for their standardized synthesis, analytical strategies for their determination in various matrices as well as the description of a variety of new biological functions.

3.1 Synthesis of vitamin E long-chain metabolites

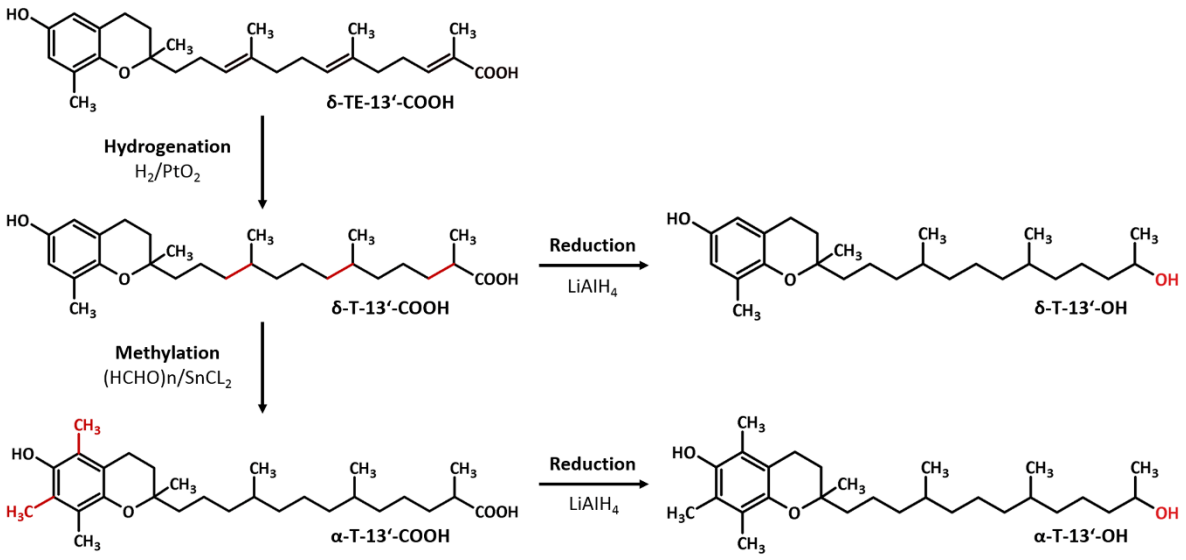

Figure 4: Semisynthesis of α-T-13'- and δ-T-13'-LCMs of vitamin E from garcinoic acid (δ-TE-13'-COOH). Changes in the structure of the respective compound after every synthesis step are marked in red. Adapted from (Birringer et al. 2010). *PtO_2* (platinum dioxide); *HCHO* (formaldehyde); *$SnCL_2$* (tin(II) chloride); *$LiAlH_4$* (lithium aluminium hydride).

The incubation of cultured cells – possessing enzymatic systems necessary for vitamin E metabolism – with TOHs or TEs represented a common method for the generation of LCMs. After sufficient incubation time, the newly formed metabolites can be obtained from the cell culture supernatant (Jiang et al. 2007; You et al. 2005; Jiang et al. 2008). However, this method has several disadvantages, since the cells produced a non-standardized mixture of LCMs, ICMs and SCMs in their conjugated or unconjugated form. Hence, it was necessary to generate the respective compounds in high purity to simplify the investigation of a single metabolite. In line

with this, Mazzini *et al.* and Birringer *et al.* developed a standardized semisynthesis for the α- and δ-LCMs from the natural compound GA (Mazzini et al. 2009; Birringer et al. 2010). Garcinoic acid or trans-δ-TE-13'-COOH can be obtained from the seeds of *Garcinia kola E. Heckel* (manuscript VI (Kluge et al. 2016)). The method for the isolation of GA was initially described by Terashima *et al.* in 1997 and has been further improved by Birringer *et al.* and Wallert *et al.* (Terashima et al. 1997; Birringer et al. 2010; Wallert et al. 2019). For a detailed description of the procedure, the reader is referred to manuscript IV. In addition to its isolation from natural origins, chemical synthesis of GA was also described (Maloney und Hecht 2005). The semisynthesis of the α- and δ-LCMs from GA is achieved in three steps **(Figure 4)**: (i) The unsaturated side chain of GA is hydrogenated in a platinum-catalyzed reaction that leads to the formation of δ-T-13'-COOH. (ii) The corresponding α-form, α-T-13'-COOH, is obtained by a $SnCl_2$ catalyzed permethylation of δ-T-13'-COOH, *i.e*. the addition of two methyl groups to the chromanol ring. (iii) The respective T-13'-OH forms can be generated via reduction of δ-T-13'-COOH or α-T-13'-COOH with $LiAlH_4$ (Mazzini et al. 2009; Birringer et al. 2010). For a detailed description of the semisynthetic route, the reader is referred to manuscript VI (Kluge et al. 2016).

3.2 Analysis of vitamin E long-chain metabolites

To date, a variety of different analytical approaches for the detection of vitamin E LCMs in cells, cell culture supernatant, human serum and plasma, tissues and feces have been published. However, the following section will only provide a short overview about the basics of LCM analysis. For more details, the reader is referred to manuscript IX (Kluge et al. 2019).

Enzymatic deconjugation of the LCMs in a sample matrix was established as the initial step in current analytical procedures, since it improves their recovery for the later analysis (Wallert et al. 2014b). The subsequent extraction of the LCMs is accomplished by liquid-liquid or solid-phase extraction (Yang et al. 2010; Wallert et al. 2014b; Ciffolilli et al. 2015; Giusepponi et al. 2017). The extracted metabolites are afterwards separated by high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to different detectors. Here, the expected LCM concentration in the respective sample is crucial for the selection of a suitable detection method. While fluorescence detection is sufficient for cell culture samples, the analysis of LCMs in human plasma or serum requires detection via mass spectrometry (MS) (Kluge et al. 2019). A subset of analyzable LCMs in human blood is provided in **Table 3**.

Challenges of LCM analysis in human blood

The detection of LCMs in human blood appeared as one of the greatest challenges of vitamin E research in the last decade. As already mentioned, serum concentrations of α-T-13'-COOH and α-T-13'-OH are at low nanomolar levels. Hence, their analysis requires high sensitive detection methods, *e.g.* LC-MS/MS or GC-MS/MS, as well as specific sample preparation procedures that increase their recovery, *i.e.* enzymatic deconjugation of the LCMs by sulfatase and β-glucuronidase (Wallert et al. 2014b). Since the initial detection of α-T-13'-COOH in human serum, analytical approaches for vitamin E LCMs were constantly improved over time

(Ciffolilli et al. 2015; Torquato et al. 2016b). The latest procedure enabled the simultaneous detection of α-T-13'-COOH and α-T-13'-OH together with their vitamin precursors in the same sample (Giusepponi et al. 2017). However, there are still problems to solve. It was recently recognized that the blood concentration of α-T-13'-COOH is probably detected as a bulk parameter comprised of up to three different isomers (Giusepponi et al. 2017). Unfortunately, the origin of these stereoisomers is currently unknown, but their separation and characterization represents a challenge for future analytical procedures, since it would shed light on the question whether the proposed systemic relevance of vitamin E LCMs depends on a specific isomer.

Table 3: Subset of analyzable LCMs in human blood with their corresponding preparation and detection methods. Adapted from (Kluge et al. 2019). *Deconj.* (deconjugation); Extr. (extraction); *G* (glucoronidase); *LC* (liquid chromatography); *LL* (liquid-liquid); *n.i.* (no information); *Q-TOF* (quadrupole time-of-flight); *S* (sulfatase)

Deconj.	Extr.	Chromatography	Sample	Metabolite	Reference
S + G	LL	Q-TOF-LC-MS	Human serum	α-T-13'-COOH	(Wallert et al. 2014b)
-	LL	HPLC-ECD GC-MS	Human serum	α-T-13'-OH	(Ciffolilli et al. 2015)
n.i.	LL	LC-MS/MS	Human serum	α-T-13'-COOH α-T-13'-OH	(Torquato et al. 2016b)
S + G	LL	LC-MS/MS	Human serum + plasma	α-T-13'-COOH α-T-13'-OH	(Giusepponi et al. 2017)
n.i.	LL	UPLC-MS/MS	Human plasma	α-T-13'-COOH	(Pein et al. 2018)

3.3 Biological activities of vitamin E long-chain metabolites

Since the initial description of an inhibitory effect of γ- and δ-T-13'-COOH on the activity of cyclooxygenase (COX)-2 in human lung carcinoma A549 cells (Jiang et al. 2008), the interest on the biological functions of vitamin E LCMs has constantly grown over the years. Today, the variety of known biological effects of the LCMs can be categorized as follows: (i) anti-inflammatory activity, (ii) anti-carcinogenic effects, (iii) regulation of cellular lipid homeostasis, (iv) interaction with pharmaceuticals, and (v) regulation of vitamin E metabolism. Since their biological activities have been extensively reviewed in the manuscripts VI to X, the following sections will only provide a compact overview about the most important effects of the LCMs. For a more detailed description, the reader is referred to the respective manuscripts.

3.3.1 Anti-inflammatory activities

Inflammation is a key factor in the pathogenesis of several non-communicable human diseases such as metabolic syndrome, diabetes mellitus, cardiovascular disease, neurodegenerative disease and cancer (Okin und Medzhitov 2012). The complex process of systemic inflammation is triggered by different key factors, including pro-inflammatory enzymes, chemokines and cytokines (Schubert et al. 2018). Hence, research on the anti-inflammatory actions of vitamin E-derived LCMs predominantly focused on these targets. To date, several LCMs, such as α-, γ-, and δ-T-13'-COOH, δ-T-9'-COOH as well as α- and δ-T-13'-OH, were shown to reduce stimulus-induced expression, activity or production of different pro-inflammatory factors (Wallert et al. 2020).

Treatment with 10 µM of either α-T-13'-OH or δ-T-13'-OH decreased the expression of inducible nitric oxide synthase (iNOS) and COX-2 in lipopolysaccharide (LPS)-stimulated murine RAW264.7 macrophages (Ciffolilli et al. 2015; Schmölz et al. 2017). In addition, interleukin (IL)-β and IL-6 mRNA expression as well as prostaglandin (PG) E_2 production was also reduced by α-T-13'-OH (10 µM) (Ciffolilli et al. 2015). The carboxy metabolites α-T-13'-COOH and δ-T-13'-COOH revealed similar effects in LPS-stimulated murine RAW264.7 macrophages, although lower concentrations (5 µM) were necessary to reduce the expression of iNOS, COX-2 and pro-IL-1β (Schmölz et al. 2017; Wallert et al. 2015). Interestingly, only δ-T-13'-COOH (5 µM) decreased the enzymatic activity of COX-2, while α-T-13'-COOH (5 - 10 µM) showed no effect (Jiang et al. 2008; Wallert et al. 2015; Pein et al. 2018). Nevertheless, α-T-13'-COOH blocked the LPS-induced production of nitric oxide and PGE_2 in murine macrophages (5 µM) (Wallert et al. 2015). Beside the mentioned effects on iNOS and COX-2, α-T-13'-COOH also reduced 5-lipoxygenase (LO) product formation, *i.e.* production of leukotriene (LT)B4), *in vitro* and *in vivo* (Pein et al. 2018). Compared with TOH-derived LCMs, the knowledge on anti-inflammatory activities of TE-derived LCMs is sparse. However, δ-TE-13'-COOH (5 µM) (*i.e.* garcinoic acid) reduced the expression of iNOS, COX-2, pro-IL-1β, IL-6 and TNF-α as well as the formation of their respective products, *i.e.* nitric oxide, PGE_2, and thromboxane (TX)B_2, in LPS-stimulated murine RAW264.7 macrophages (manuscript IV (Wallert et al. 2019)). In addition, PGE_2 formation in LPS-stimulated monocytes was already inhibited at a δ-TE-13'-COOH concentration of 300 nM (Pein et al. 2018).

3.3.2 Anti-carcinogenic effects

Abnormal proliferation together with a reduced rate of apoptosis are two major characteristics of cancer cells. Thus, current cancer therapies predominantly rely on drugs that are able to kill cells with high rates of proliferation and regeneration. Interestingly, natural compounds with anti-proliferative or pro-apoptotic activities are regarded as beneficial in the prevention and treatment of cancer, as they generally exert less side effects compared with conventional drugs (Schubert et al. 2018). To date, several natural compounds, including the LCMs of vitamin E, have been identified to affect key factors of cancer development.

In an investigation in the human liver cancer cell line HepG2, α-T-13'-COOH (20 µM) and δ-T-13'-COOH (20 µM) caused cell growth arrest, while their corresponding 13'-OH metabolites

did not affect cell proliferation. Further, α- and δ-T13'-COOH as well as δ-T-13'-OH reduced cell viability of HepG2 cells via induction of pro-apoptotic pathways, indicated by an enhanced cleavage of poly[ADP-ribose]-polymerase 1 and caspases 3, 7 and 9 (Birringer et al. 2010). The observed pro-apoptotic effects of α-T-13'-COOH and δ-T-13'-COOH were confirmed in human leukemia-derived THP-1 macrophages (IC_{50} of 7.4 µM or 11.1 µM respectively) (Wallert et al. 2014b; Schmölz et al. 2017). In addition, anti-proliferative and pro-apoptotic effects of δ-T-13'-COOH were also described in human HCT-116 colon carcinoma cells (IC_{50} 8.9 µM) and human HT-29 colorectal adenocarcinoma cells (IC_{50} 8.6 µM) (Jang et al. 2016). So far, δ-TE-13'-COOH (*i.e.* garcinoic acid) is the only TE-derived LCM with a known anti-carcinogenic potential. Garcinoic acid decreased the viability of human HCT-116 colon carcinoma cells, human HT-29 colorectal adenocarcinoma cells (Jang et al. 2016) and human glioma C6 cells (Mazzini et al. 2009) as well as colon tumor growth in BALB/c mice (Jang et al. 2016).

3.3.3 Effects on lipid homeostasis

Only a few modulatory activities of the LCMs on different facets of the cellular lipid homeostasis have been described so far. These include regulation of CD36 and regulation of perilipin 2 (PLIN2), better known as adipophilin, mediation of the uptake of oxidized low-density lipoproteins (oxLDL), phagocytosis as well as intracellular storage of lipids. Overall, these results indicate that the LCMs could be involved in the regulation of macrophage foam cell formation, a hallmark in the pathogenesis of atherosclerosis.

The LCMs α-T-13'-COOH (5 µM) and α-T-13'-OH (10 µM) induced CD36 expression in human leukemia-derived THP-1 macrophages and primary macrophages (Wallert et al. 2014b). In addition, ox-LDL induced CD36 expression – a pathophysiologic positive feedback loop in macrophage foam cell formation – was augmented by α-T-13'-COOH and α-T-13'-OH. Surprisingly, oxLDL uptake into THP-1 macrophages decreased after pre-incubation with either α-T-13'-COOH (20%) or α-T-13'-OH (24%). In line with this, cellular neutral lipid accumulation was also not increased. These contradictory results were at least in parts explained by a reduction of oxLDL uptake via phagocytosis after LCM treatment (Wallert et al. 2014b). Besides their interaction with oxLDL uptake, LCMs also affected intracellular lipid storage. Hence, α-T-13'-COOH (5 µM) induced the expression of PLIN2 as well as the size and number of intracellular lipid droplets in human THP-1 macrophages. Further, α-T-13'-COOH was able to protect THP-1 cells from stearic acid induced lipotoxicity, which was at least in parts attributed to the upregulation of PLIN2 expression (Schmölz et al. 2018). In addition to the already existing knowledge about the interference of α-T-13'-COOH with the cellular lipid homeostasis, manuscript I provides new insights into another aspect of the regulatory role of α-T-13'-COOH on macrophage foam cell formation. We here show for the first time that α-T-13'-COOH (5 µM) is able to protect human THP-1 macrophages against excessive lipid accumulation in the presence of VLDL oversupply. The protective effect resulted from a very potent upregulation of angiopoietin-like (ANGPTL) 4 mRNA expression, which in turn decreased the LPL mediated uptake of lipids. Thus, manuscript I provides further evidence for an atheroprotective role of α-T-13'-COOH.

3.3.4 Other known biological effects

3.3.4.1 Interaction with pharmaceuticals

The expression of P-glycoprotein (P-gp) – a protein that regulates the intracellular concentration of pharmaceuticals – is among others regulated by the pregnane X receptor (PXR) (Dewanjee et al. 2017). Interestingly, α-T-13'-COOH enhanced P-gp expression in human epithelial-like colon LS180 cells as well as the activity of PXR in a reporter gene assay (Podszun et al. 2017). In addition, δ-TE-13'-COOH (*i.e.* garcinoic acid) was also proposed as a selective PXR agonist (Bartolini et al. 2020).

3.3.4.2 Regulation of LCM formation

So far, the regulatory mechanisms modulating the metabolism of vitamin E are still unknown. However, Torquato *et al.* showed that the expression of CYP4F2 – the rate limiting enzyme in vitamin E metabolism – was upregulated by α-T-13'-OH in human HepG2 cells (Torquato et al. 2016a). This result provides evidence for a positive regulatory feedback loop of the LCMs on the metabolism of vitamin E. Interestingly, the positive feedback regulation would be in contrast to other fat-soluble vitamins, as the metabolism of vitamin A and D is negatively regulated by their metabolites (Schubert et al. 2018).

3.3.4.3 Structure specific effects

As already indicated, the different vitamin E forms and LCMs seem to reveal their biological activities with different efficiencies. To test this hypothesis, Schmölz *et al.* used the α- and δ-forms of T-13'-OH and T-13'-COOH together with α- and δ-TOH to investigate their regulatory potential on CD36 and iNOS expression. Further, two additional substances representing specific substructures of the LCMs, *i.e.* the SCM α-CEHC mimicking the chromanol ring system as well as pristanic acid imitating the modified side chain, were also included in the investigation. All investigated LCMs revealed potent regulatory effects on the expression of CD36 and iNOS even at low concentrations. In contrast, neither their vitamin precursors nor the included substructures were able to accomplish comparable effects. Overall, the 13'-COOH metabolites showed the most potent effects of all investigated compounds (Schmölz et al. 2017).

4 Atherosclerosis

Although most of the current studies on vitamin E-derived LCMs are focused on one specific functional aspect, the entirety of known biological activities can be assigned to different human diseases. For example, LCMs are involved in the regulation of inflammation and the cellular lipid homeostasis (especially in macrophages), two key factors for the pathogenesis of atherosclerosis. Atherosclerosis together with its complications stroke and myocardial infarction are by far the leading cause of death in industrialized Western societies (Cheng et al. 2020). Since atherosclerotic events increased over the last decades, their development seems to correlate with different factors of the modern lifestyle. This includes food oversupply and quality, lack of physical activity and smoking (Rafieian-Kopaei et al. 2014). The

development and progression of atherosclerosis is characterized by a complex interaction of different processes over a long time. In brief, different endogenous and exogenous noxes cause an initial endothelial dysfunction that leads to intimal thickening due to the recruitment of T-cells and monocytes into the subintimal space. The pathogenesis continues due various events, such as differentiation of monocytes to macrophages, lipid accumulation in macrophages as well as proliferation of lipid-loaded macrophages, deposition of cholesterol and fibrotic material, foam cell formation, fatty streak formation, elasticity loss and reduction of the vascular lumen with parallel widening of the arterial diameter. Finally, atherosclerotic changes of the intima lead to the formation of a necrotic lipid core with a fibrotic cap. Continuous destabilization of the fibrous cap by proteases results in plaque rupture and thrombus formation. Subsequently, the thrombus flows with the bloodstream and causes vascular occlusion in an artery with a smaller volume. The thrombus can also be integrated in the plaque, leading to the formation of a so-called complicated plaque (Wallert et al. 2014c).

The pathogenesis of atherosclerosis as well as the potential therapeutic effect of vitamin E in large human intervention trials have been extensively reviewed by our group (the reader is referred to (Wallert et al. 2014c)). In addition, our previous investigations provided evidence for an association between the vitamin E-derived LCMs and classic key factors in the development of atherosclerosis, *i.e.* inflammation and oxLDL triggered foam cell formation (Wallert et al. 2014b; Wallert et al. 2015). The following section will therefore focus on a different aspect in the development of atherosclerosis, *i.e.* the triglyceride-rich lipoprotein (TRL) mediated foam cell formation. In line with this, manuscript I provides convincing evidence for a regulatory role of the vitamin E LCM α-T-13'-COOH in this underestimated part of the atherosclerotic process.

4.1 The relevance of the forgotten lipids for atherosclerosis

Elevated low-density lipoprotein (LDL) cholesterol is a well-established risk factor for the development of atherosclerosis. According to national and international guidelines, LDL cholesterol represents a key therapeutic target for the prevention and treatment of atherosclerosis (Stone et al. 2014; Mach et al. 2020). In line with this, LDL-lowering therapies, such as treatment with statins, ezetimibe, and proprotein convertase subtilisin-kexin type 9 inhibitors, have been introduced in clinical practice (Cannon et al. 2015; Sabatine et al. 2017). However, despite of the significant reduction of LDL cholesterol levels by lipid-lowering agents, patients still reveal recurrent cardiovascular events (Sampson et al. 2012; Madsen et al. 2018). Interestingly, results from several large-scale epidemiological studies provided evidence for a casual association between TRLs, *i.e.* chylomicrons and VLDL, and atherosclerosis (Nordestgaard 2016). Outcomes from genetic studies further strengthened this concept. Here, genetic modification of key genes in TRL metabolism, such as *Lpl*, apolipoprotein (*Apo*) *A-V*, *ApoC-III* and *Angptl4*, strongly correlated with an increased risk for atherosclerotic events (Musunuru und Kathiresan 2016). Since TRLs represent the predominant carriers of triglycerides in blood, their concentration directly correlates with serum triglyceride level. Therefore, determination of serum triglycerides represents a valid

and established biomarker for the detection of enhanced TRL levels in humans. In accordance with the guidelines of the American Herat Association, moderate hypertriglyceridemia is defined by a fasting serum triglyceride level of ≥ 150 mg/dL (1.7 mmol/L) (Miller et al. 2011). The moderate elevation of serum triglycerides is predominantly promoted by lifestyle factors, such as overweight, diets enriched in saturated fats and carbohydrates as well as excess alcohol consumption. Based on the results of national surveys, moderate hypertriglyceridemia was present in 32% of the American and in 27% of a comparable European population (Miller et al. 2011; Mortensen et al. 2015). Hence, moderate hypertriglyceridemia affects a significant part of the population, indicating the putative relevance of TRLs for the development of atherosclerosis in Western societies. Besides triglycerides, TRLs carry varying amounts of cholesterol, the so-called remnant cholesterol. The cholesterol amount of TRLs – especially of VLDL – was also considered as a crucial factor in TRL mediated development of atherosclerosis (Nordestgaard 2016).

4.2 Mechanisms of TRL mediated development of atherosclerosis

It was demonstrated that VLDL is able to penetrate the arterial wall to enter the intima, *i.e.* the site of plaque formation (Shaikh et al. 1991; Nordestgaard et al. 1992). In line with this, VLDL was detected as the predominant form of TRLs in atherosclerotic plaques (Nakano et al. 2008). It is supposed that after VLDL entered the arterial wall, the lipoprotein particle is trapped by proteoglycans or other components of the intima, as it was already shown for LDL (Borén et al. 1998). Based on the available data, VLDL mediated atherosclerotic changes in the intima are probably promoted by two major mechanisms: (i) The triglycerides of the VLDL particles are degraded by the activity of LPL on the endothelial surface or the surface of macrophages and foam cells in atherosclerotic plaques (O'Brien et al. 1992; Kersten 2014). This leads to the liberation of free fatty acids that on the one hand cause local inflammation (Rosenson et al. 2014) and on the other hand promote macrophage foam cell formation (Skarlatos et al. 1993). (ii) Unlike LDL particles that require additional oxidative modification, VLDL particles can be directly taken up by macrophages due to the VLDL receptor (Miller et al. 2010). The VLDL receptor functions as a peripheral lipoprotein receptor in concert with LPL, especially on the surface of macrophages (Takahashi et al. 2004; Takahashi 2017). Here, cholesterol content of VLDL seems to be the crucial factor for foam cell formation, like it has been shown for the uncontrolled uptake of oxLDL (Nordestgaard und Varbo 2014). Interestingly, some investigation suggested VLDL to reveal an even higher pro-atherogenic potential than LDL because of its facilitated uptake via the VLDL receptor (Toth 2016). Since both suggested mechanism directly or indirectly involve LPL, the LPL system (extensively reviewed in (Dijk und Kersten 2016)) seems to play a major role in TRL mediated development of atherosclerosis. This concept is further strengthened by the outcomes of a recent genetic study, showing that mutations of key genes in the LPL system, i.e. *LPL* and A*NGPTL4* were correlated with an increased risk for atherosclerosis (Musunuru und Kathiresan 2016; Kersten 2019). If this concept holds true, the LPL system would appear as a new target in the

prevention of atherosclerosis. A schematic overview of the general mechanisms in LPL promoted foam cell formation is provided in **Figure 5**.

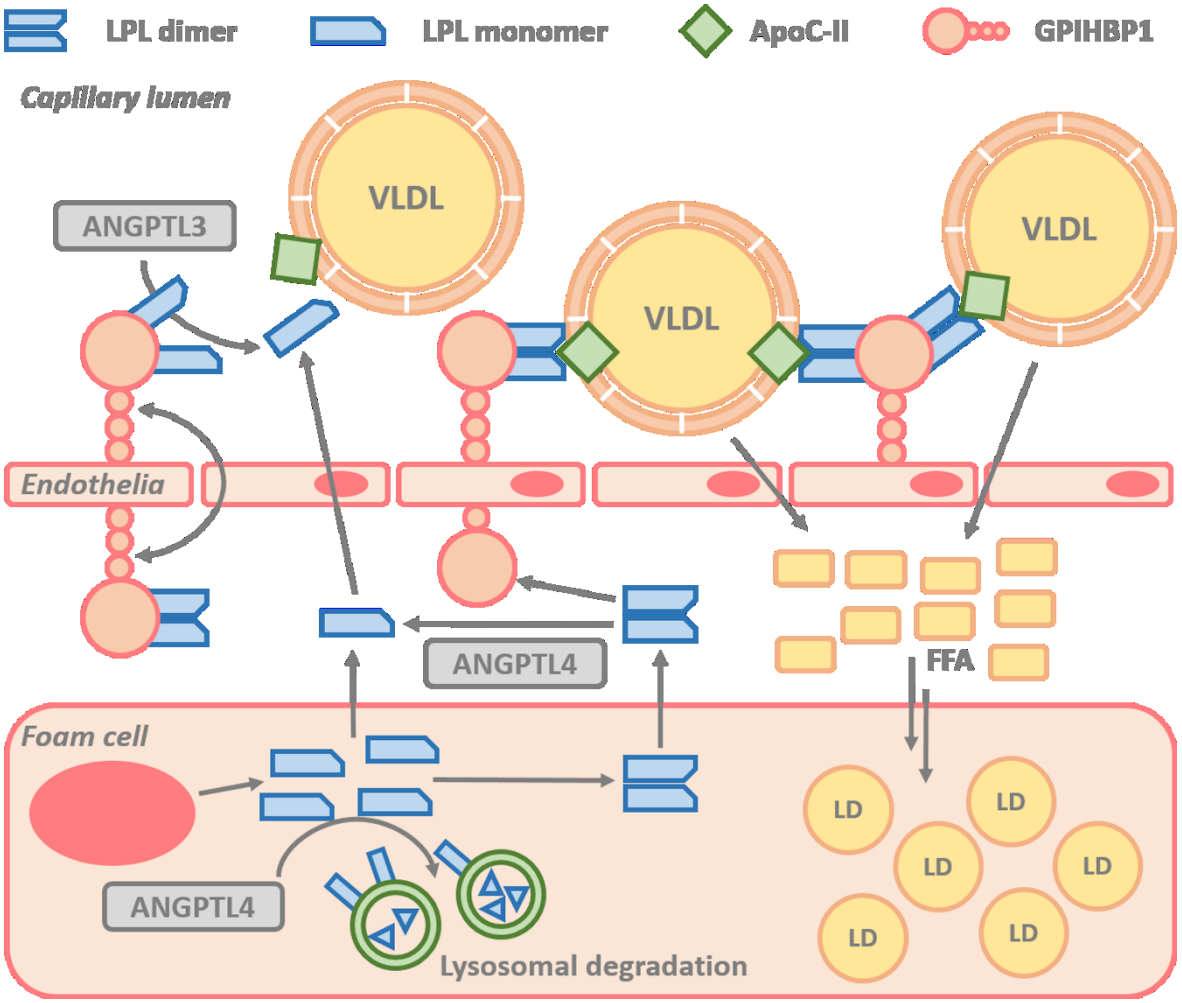

Figure 5: The LPL pathway and its potential involvement in foam cell formation and atherosclerosis. Adapted from (Tada et al. 2018). *ANGPTL* (angiopoietin-like), *ApoC-II* (apo protein C-II), *FFA* (free fatty acid), *GPIHBP1* (glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1), *LD* (lipid droplet), *LPL* (lipoprotein lipase), *VLDL* (very low density lipoprotein). Functional LPL homodimers are transferred to the endothelial surface where they bind to GPIHBP1. Here, functional LPL hydrolyzes the triglycerides of VLDL particles, which leads to the liberation of FFAs. In turn, absorption of high amounts of FFAs by macrophages promotes foam cell formation. Physiological regulator proteins, such as ANGPTL3 and ANGPTL4, are able to inhibit LPL activity, while other proteins, such as ApoC-II, promote LPL activity. Since it was shown that mutations of key genes in the LPL system, *i.e. LPL* and *ANGPTL4,* are associated with an increased risk for atherosclerosis, the LPL system seems to play a major role in VLDL-mediated atherogenesis.

5 Aim of the thesis

Almost one hundred years after its discovery in 1922, the function of vitamin E in humans is still not elucidated. In a recent perspective on the occasion of the 100th anniversary of the discovery of vitamin E, the current state of research was summarized as follows: "[...] we have still more questions than answers regarding the biological functions and the essentiality of vitamin E for human health" (Galli et al. 2017). Nevertheless, vitamin E research made great progress over the last 100 years and is currently experiencing a scientific 'renaissance', which is among others attributed to the discovery of vitamin E metabolism in humans as well as the elucidation of biological functions of the formed metabolites. Especially since the determination of vitamin E LCMs in human serum – indicating their systemic relevance in the human body – it is becoming more and more evident that the essentiality of vitamin E for human health cannot be explained likely without the consideration of its metabolites. Thus, renowned scientist on the field hypothesized that the LCMs are not only side-products of vitamin E excess, but may represent activated and therefore functional molecules of their vitamin precursors (Galli et al. 2017).

Hence, the major aim of this PhD thesis was to increase our knowledge on the function of vitamin E metabolites as well as to generate a significant contribution to the progress of this promising new facet of vitamin E research. In more detail, the thesis predominantly focused on four aims:

(i) The current knowledge about vitamin E-derived LCMs together with emerging aspects for future research should be provided to a broad scientific community due to the preparation of different review articles on their biological functions, chemical analysis and potential benefit for human health. This should contribute to increase the interest in this new research topic as well as to promote the generation of new working hypotheses for future studies.

(ii) The elucidation of additional regulatory functions of the vitamin E-derived LCMs in lipid metabolism and inflammation. The aim of these investigations was to deepen our insights into the modes of action of the LCMs as well as to derive potential functions relevant for human health, especially for atherosclerosis. This did also include the development of new methodical approaches, facilitating the investigation of different aspects of the LCM function.

(iii) The generation of data further strengthening the concept that the vitamin E-derived LCMs represent functional molecules of their vitamin precursors. This should be achieved by the parallel investigation of LCMs and their respective vitamin precursor *in vitro*. If the concept holds true, this would also provide evidence for a general mechanism of metabolic activation for fat-soluble vitamins, as it was already described for the vitamins A and D (Schubert et al. 2018).

(iv) The generation of convincing *in vitro* data, that justify future studies on the indicated systemic relevance of the vitamin E LCMs *in vivo*.

6 Manuscript overview

The following section provides an overview about the publications and manuscripts included in this thesis. All publications deal with the topic of vitamin E and vitamin E-derived LCMs and were created by myself or in collaboration with me. A graphical overview about the thematic linkage of the individual publications together with a statement of my personal contribution to the respective manuscript (percentage) is provided in **Figure 6**.

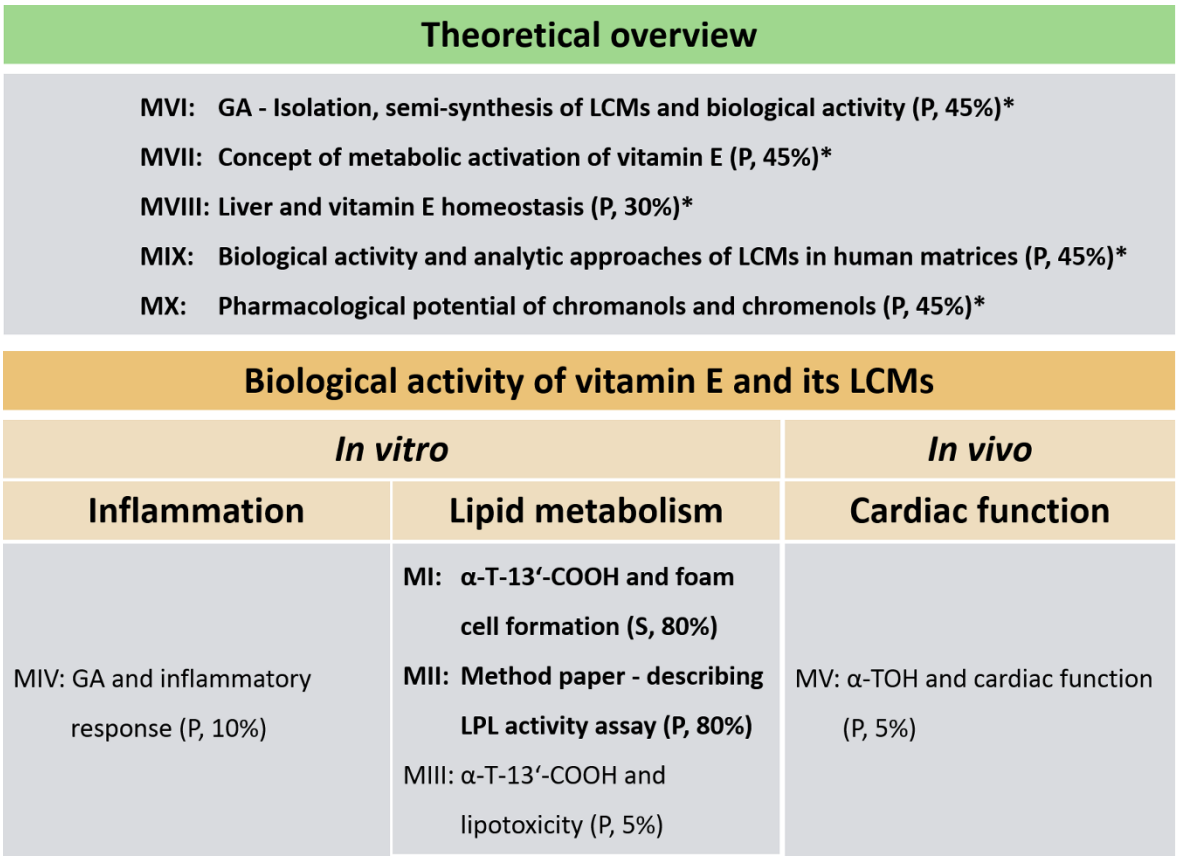

Figure 6: Schematic overview about the manuscripts included the thesis. Manuscripts marked in bold are first authorships. My personal contribution to the respective manuscript is stated as percentage. *GA* (garcinoic acid), *LCM* (long-chain metabolite), *M* (manuscript), *P* (published), *S* (submitted), * (shared authorship).

Manuscript I

The vitamin E long-chain metabolite α-13'-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system

Stefan Kluge, Martin Schubert, Lisa Börmel, Stefan Lorkowski

BBA Molecular and Cell Biology of Lipids

DOI: 10.1016/j.bbalip.2021.158875

Date of acceptance: 2nd January 2021

Contribution to the manuscript

- Concept development
- Performance of lipoprotein isolation, RT-qPCRs, western blots, measurements of lipoprotein lipase activity, analysis of intracellular neutral lipid accumulation
- Data analysis
- Data evaluation
- Drafting of the manuscript

Personal contribution to the manuscript: 80%

Key messages of manuscript I:

The manuscript provides new insights into the regulatory role of α-T-13-COOH and its vitamin precursor α-TOH on VLDL-triggered foam cell formation. Both compounds affected the LPL system by different regulatory modes. While α-TOH mediated LPL expression via transcriptional regulation, α-T-13'-COOH triggered post-translational regulation of LPL via ANGPTL4. Further, both compounds effectively reduced catalytic activity of LPL. However, only α-T-13'-COOH was able to protect human THP-1 macrophages against excessive lipid accumulation in presence of VLDL oversupply. In addition, much higher doses of α-TOH (100 µM) were required to preserve regulatory effects compared to α-T-13'-COOH (5 µM). Although their exact mode action is still unknown, our results support the concept that LCMs of vitamin E, including α-T-13'-COOH, represent activated and therefore functional molecules of their vitamin precursors. Since only α-T-13'-COOH and not α-TOH prevented excessive lipid accumulation in macrophages, we provide further evidence for an atheroprotective potential of this regulatory metabolite.

Manuscript II

Simple and rapid real-time monitoring of LPL activity in vitro

Stefan Kluge, Lisa Boermel, Martin Schubert, Stefan Lorkowski

MethodsX (2020):7;100865

DOI: 10.1016/j.mex.2020.100865

Date of acceptance: 10th March 2020

Contribution to the manuscript

- Concept development
- Performance of lipoprotein isolation, RT-qPCRs, measurements of lipoprotein lipase activity, analysis of intracellular neutral lipid accumulation
- Data analysis
- Data evaluation
- Drafting of the manuscript

Personal contribution to the manuscript: 80%

Key messages of manuscript II:

This research article provides a step-by-step protocol of a new fluorescence-based assay for the *in vitro* assessment of lipoprotein lipase activity (LPL). The assay protocol was validated by different experimental settings, using the well-characterized peroxisome proliferator-activated receptor (PPAR)δ agonist GW0742 as test compound. The assay has several advantages over currently available *in vitro* LPL assays: (i) 12-well cell culture plate design for the simultaneous investigation of up to three different test compounds (including all assay controls); (ii) 24 h real-time acquisition of LPL activity data for the identification of the optimal timepoint for further measurements; (iii) LPL activity measurement can be complemented by additional cell and molecular biological analyses using the same cell samples. In our hands, the described assay design can serve as a reliable tool for *in vitro* measurements of the LPL system.

Manuscript III

Long-chain metabolites of vitamin E: Interference with lipotoxicity via lipid droplet associated protein PLIN2

Lisa Schmölz, Martin Schubert, Jasmin Kirschner, **Stefan Kluge**, Francesco Galli, Marc Birringer, Maria Wallert, Stefan Lorkowski

BBA Molecular and Cell Biology of Lipids (2018):1863(8);919-927

DOI: 10.1016/j.bbalip.2018.05.002

Date of acceptance: 3rd May 2018

Contribution to the manuscript

- Critical evaluation of the manuscript
- Discussion and evaluation of data/results

Personal contribution to the manuscript: 5%

Key messages of manuscript III:

This research article provides evidence for a protective effect of α-LCMs against stearic acid-induced lipotoxicity in human THP-1 macrophages. The protective effect was linked to the increase of lipid storage capacity at least in parts due to the induction of PLIN2 expression. In contrast, the vitamin precursor α-TOH induced lipotoxicity already under basal conditions without the addition of stearic acid. This data further strengthens the concept of a metabolic activation of vitamin E to functional molecules (LCMs), revealing more potent or even different effects compared to their vitamin precursors.

Manuscript IV

The vitamin E derivative garcinoic acid from Garcinia kola nut seeds attenuates the inflammatory response

Maria Wallert, Julia Bauer, **Stefan Kluge**, Lisa Schmölz, Yung-Chih Chen, Melanie Ziegler, Amy Searlea, Alexander Maxones, Martin Schubert, Maria Thürmer, Helmut Pein, Andreas Koeberle, Oliver Werz, Marc Birringer, Karlheinz Peter, Stefan Lorkowski

Redox Biology (2019):24;101166

DOI: 10.1016/j.redox.2019.101166

Date of acceptance: 10th March 2019

Contribution to the manuscript

- Implementation of western blots
- Data analysis
- Data evaluation
- Critical evaluation of the manuscript

Personal contribution to the manuscript: 10%

Key messages of manuscript IV:

This research article investigated the anti-inflammatory and anti-atherogenic potential of garcinoic acid (δ-TE-13'-COOH), a phytochemical obtained from the seeds of *garcinia kola E. Heckel*, in LPS-activated mouse RAW264.7 macrophages and $Apoe^{-/-}$ mice respectively. It was shown that GA efficiently reduced LPS-induced inflammation via inhibition of pro-inflammatory pathways *in vitro*. However, weekly administration of GA did not affect inflammation and atherosclerotic events in $Apoe^{-/-}$ mice. Hence, the promising anti-inflammatory effects of GA observed *in vitro* were not reproducible in an *in vivo* model of chronic low grade inflammation.

Manuscript V

α-Tocopherol preserves cardiac function by reducing oxidative stress and inflammation in ischemia/reperfusion injury

Maria Wallert, Melanie Ziegler, Xiaowei Wang, Ana Maluenda, Xiaoqui Xuc, May Lin Yap, Roman Witt, Corey Giles, **Stefan Kluge**, Marcus Hortmann, Jianxiang Zhang, Peter Meikle, Stefan Lorkowski, Karlheinz Peter

Redox Biology (2019):26;101292

DOI: 10.1016/j.redox.2019.101292

Date of acceptance: 5th August 2019

Contribution to the manuscript

- Implementation of HPLC analysis of α-tocopherol in mouse plasma
- Data analysis
- Data evaluation
- Critical evaluation of the manuscript

Personal contribution to the manuscript: 5%

Key messages of manuscript V:

This research article investigated the potential of an acute α-TOH administration as a new therapeutic strategy for the treatment of cardiac damage after myocardial infarction in a murine cardiac ischemia/reperfusion injury model. Administration of α-TOH significantly reduced infarct size, cardiac output, stroke volume and restored cardiac function. The observed cardioprotective effect of α-TOH was, among others, linked to decreased infiltration of neutrophils in the cardiac tissue as well as to reduced amounts of reactive oxygen species and lipid peroxidation markers within the infarcted tissue. The study provided strong evidence for the therapeutic use of vitamin E as an acute treatment for patients who suffer myocardial infarction.

Manuscript VI

Garcinoic acid: A promising bioactive natural product for better understanding the physiological functions of tocopherol metabolites

Stefan Kluge*, Martin Schubert*, Lisa Schmölz, Marc Birringer, Maria Wallert, Stefan Lorkowski

*authors contributed equal

Studies in Natural Products Chemistry (2016):51;435-481.

DOI: 10.1016/B978-0-444-63932-5.00009-7

Date of acceptance: 24th February 2016

Contribution to the manuscript

- Creation of the concept
- Investigation and evaluation of data
- Drafting of the manuscript

Personal contribution to the manuscript: 45%

Key messages of manuscript VI:

This review article focusses on the natural sources, the chemical extraction and the biological effects of GA (δ-TE-13'-COOH), a natural compound of the African bitternut Garcinia kola, with great significance for traditional ethno medicine. The publication also attends the great importance of GA for *in vitro* and *in vivo* investigation of vitamin E-derived LCMs, since these metabolites can be generated via semi-synthesis from GA. In addition, the article provides a comprehensive overview about known biological functions of TOHs and TEs together with their respective metabolites, focusing on anti-oxidant-, anti-inflammatory and anti-carcinogenic effects.

Manuscript VII

Long-chain metabolites of vitamin E: Metabolic activation as a general concept for lipid-soluble vitamins?

Martin Schubert*, **Stefan Kluge***, Lisa Schmölz, Maria Wallert, Francesco Galli, Marc Birringer, Stefan Lorkowski

*authors contributed equal

Antioxidants (2018):7(1);10

DOI: 10.3390/antiox7010010

Date of acceptance: 11th January 2018

Contribution to the manuscript

- Creation of the concept
- Investigation and evaluation of data
- Drafting of the manuscript

Personal contribution to the manuscript: 45%

Key messages of manuscript VII:

This review article provides an overview about the intestinal absorption, distribution and metabolization of the fat-soluble vitamins A, D, E and K. Further, known biological activities of their respective metabolites are described under the perspective of a general concept of metabolic activation of fat-soluble vitamins. In the case of vitamin E, detection of vitamin E-derived LCMs in human serum indicated a potential systemic relevance of these metabolites as a new class of regulatory molecules. This concept was strengthened by several studies, revealing anti-oxidative, anti-inflammatory, anti-carcinogenic and to some extend anti-atherogenic effects of tocopherol-derived LCMs. Interestingly, LCMs revealed more potent and in part even contrary effects to their vitamin precursors. Therefore, the LCMs must be taken into consideration to correctly interpret the effects of vitamin E in humans.

Manuscript VIII

The hepatic fate of vitamin E

Lisa Schmölz*, Martin Schubert*, **Stefan Kluge***, Marc Birringer, Maria Wallert, Stefan Lorkowski

*authors contributed equal

Vitamin E in Health and Disease (2018):1;1-30

DOI: 10.5772/intechopen.79445

Date of acceptance: 5th November 2018

Contribution to the manuscript

- Creation of the concept
- Investigation and evaluation of data
- Drafting of the manuscript

Personal contribution to the manuscript: 30%

Key messages of manuscript VIII:

This review article focuses on the liver as the central organ of the human vitamin E homeostasis. On the one hand, the liver accomplishes the physiologic handling of vitamin E, including uptake, distribution, metabolism and storage. On the other hand, the liver is also a starting point for the modulation of vitamin E homeostasis by physiological, non-physiological and pathophysiological factors. As a prominent example, the neurological disorder ataxia with vitamin E deficiency (AVED) is linked to a mutation in the gene encoding for α-TTP, a protein responsible for the hepatic discrimination of α-TOH from the other forms of vitamin E. Beside its significance for the handling of vitamin E, the liver is also the key organ for the formation of vitamin E-derived LCMs.

Manuscript IX

Bioactivity of vitamin E long-chain metabolites

Stefan Kluge*, Martin Schubert*, Lisa Schmölz, Maria Wallert, Marc Birringer, Stefan Lorkowski

*authors contributed equal

Vitamin E in Human Health (2019):96;61-79 Springer Nature

DOI: 10.1007/978-3-030-05315-4_6

Date of acceptance: 11th March 2019

Contribution to the manuscript

- Creation of the concept
- Investigation and evaluation of data
- Drafting of the manuscript

Personal contribution to the manuscript: 45%

Key messages of manuscript IX:

This review article focuses on the formation, distribution and regulatory properties of vitamin E LCMs in humans. A special focus of this article is on the description of analytical approaches for the detection of LCMs in human matrices. The analysis of LCMs in humans is of great importance to elucidate their suggested systemic relevance as a new class of signaling molecules. However, LCM analysis in human matrices is challenging and existing strategies for vitamin E analysis required further improvement to enable a reliable analysis of these compounds. The adaptations included an additional enzymatic deconjugation step for sample preparation as well as the use of more accurate techniques for sample separation and detection. Although the analysis of the LCMs made great progress over the last decades, especially the distribution of the LCMs in extrahepatic tissues beside human serum requires further investigation.

Manuscript X

Diversity of chromanol and chromenol structures and functions: an emerging class of anti-inflammatory and anti-carcinogenic agents

Maria Wallert*, **Stefan Kluge***, Martin Schubert, Andreas Koeberle, Oliver Werz, Marc Birringer, Stefan Lorkowski

*authors contributed equal

Frontiers in Pharmacology (2020):11;362

DOI: 10.3389/fphar.2020.00362

Date of acceptance: 10th March 2020

Contribution to the manuscript

- Creation of the concept
- Investigation and evaluation of data
- Drafting of the manuscript

Personal contribution to the manuscript: 45%

Key messages of manuscript X:

This review article provides a comprehensive overview of the available data on signaling pathways involved in inflammation, apoptosis, cell proliferation and carcinogenesis of selected natural occurring chromanols and chromenols. Due to their ability to bind or to interfere with molecular targets and pathways, including 5-LO, nuclear receptors and the NF-κB pathway, chromanols and chromenols may represent interesting lead structures for the development of therapeutic approaches. Interestingly, the efficiency by which chromanols and chromenols exert their biological effects strongly depend on the respective regulatory target. Therefore, low- and high-affinity molecular targets of the LCMs can be classified.

7 Manuscripts

7.1 Manuscript I

BBA - Molecular and Cell Biology of Lipids 1866 (2021) 158875

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

The vitamin E long-chain metabolite α-13′-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system

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ARTICLE INFO

Keywords:
Vitamin E
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ANGPTL4
Lipoprotein lipase
Foam cell formation

ABSTRACT

The α-tocopherol-derived long-chain metabolite (α-LCM) α-13′-carboxychromanol (α-13′-COOH) is formed via enzymatic degradation of α-tocopherol (α-TOH) in the liver. In the last decade, α-13′-COOH has emerged as a new regulatory metabolite revealing more potent or even different effects compared with its vitamin precursor α-TOH. The detection of α-13′-COOH in human serum has further strengthened the concept of its physiological relevance as a potential regulatory molecule. Here, we present a new facet on the interaction of α-13′-COOH with macrophage foam cell formation. We found that α-13′-COOH (5 μM) increases angiopoietin-like 4 (ANGPTL4) mRNA expression in human THP-1 macrophages in a time- and dose-dependent manner, while α-TOH (100 μM) showed no effects. Interestingly, the mRNA level of lipoprotein lipase (LPL) was not influenced by α-13′-COOH, but α-TOH treatment led to a reduction of LPL mRNA expression. Both compounds also revealed different effects on protein level: while α-13′-COOH reduced the secreted amount of LPL protein via induction of ANGPTL4 cleavage, i.e. activation, the secreted amount of LPL in the α-TOH-treated samples was diminished due to the inhibition of mRNA expression. In line with this, both compounds reduced the catalytic activity of LPL. However, α-13′-COOH but not α-TOH attenuated VLDL-induced lipid accumulation by 35%. In conclusion, only α-13′-COOH revealed possible antiatherogenic effects due to the reduction of VLDL-induced foam cell formation in THP-1 macrophages. Our results provide further evidence for the role of α-13′-COOH as a functional metabolite of its vitamin E precursor.

1. Introduction

Almost 100 years after its initial discovery and classification as a vitamin in 1922, the biological functions and the benefit of vitamin E for human health remain a matter of debate [1]. On the occasion of this 100th anniversary in 2022, a new perspective on vitamin E and its metabolism has recently been postulated by a group of renowned scientists in the field [2]. It is suggested that especially the long-chain metabolites (LCMs) – the first metabolites formed in vitamin E metabolism – are not only side-products of vitamin E excess formed for excretion, but may represent activated and therefore functional molecules of their vitamin precursors [2]. This concept seems plausible, as it has already been accepted for other fat soluble vitamins like vitamins A and D [3–5].

The group of vitamin E is formed by eight compounds with high structural similarity, comprising a chromanol ring system linked to an aliphatic chain. Based on the saturation of the side chain, compounds are divided in tocopherols (TOHs) (saturated side chain) and tocotrienols (T3s) (unsaturated side chain). Furthermore, classification as α-, β-, γ- and δ-forms of TOHs and T3s is determined by the methylation pattern of the chromanol ring system. Tocopherols and T3s are metabolized in the liver, with α-TOH possessing the lowest catabolic rate of all vitamin

Abbreviations: 13′-COOH, 13′-carboxychromanol; 13′-OH, 13′-hydroxychromanol; ANGPTL, angiopoietin-like; CD36, cluster of differentiation 36; CYP, cytochrome P450; FBS, fetal bovine serum; FFA, free-fatty acid; FI, fluorescence intensity; GIP, glucose-dependent insulinotropic polypeptide; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1; HSPG, heparan sulfate proteoglycan; LCM, tocopherol-derived long-chain metabolite; LPL, lipoprotein lipase; LRP, low-density lipoprotein receptor-related protein; PBMC, peripheral blood mononuclear cells; PCSK, pro-protein convertase subtilisin/kexin; PPAR, peroxisome proliferator-activated receptor; PLIN2, perilipin-2; RFU, relative fluorescence units; RPMI, Roswell Park Memorial Institute; RT-qPCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; T3, tocotrienol; TOH, tocopherol; VLDL, very low-density lipoproteins.

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1388-1981/

E forms [6]. Although the exact mechanisms of metabolic degradation remain poorly understood, it is generally accepted that oxidative modification of the side chain via cytochrome P450 (CYP)-dependent enzymes (CYP4F2 and CYP3A4) leads to the formation of the LCMs 13′-hydroxychromanol (13′-OH) and 13′-carboxychromanol (13′-COOH) (extensively reviewed in [5,6]).

Interestingly, only α-TOH seems to have vitamin property due to its ability to preserve fertility in rats as well as preventing *ataxia with vitamin E deficiency* (AVED) in humans, making it the most important form of vitamin E [7]. The health-promoting effects of α-TOH were traditionally attributed to its strong antioxidant potential, although more recent investigations have also revealed non-antioxidant functions, like the modulation of gene expression and enzyme activities (reviewed in [2]). However, in the last decade, α-13′-COOH has emerged as a new regulatory metabolite, revealing more potent or even different effects compared with its vitamin precursor (reviewed in [5]). The detection of α-13′-COOH in human serum has further strengthened the concept of its physiological relevance as a potential regulatory molecule [8]. Although the biological function of α-13′-COOH is far from being unraveled, several studies have shown an interaction with inflammation [9], cancer [10] and the handling of pharmaceuticals [11]. In addition, two recent investigations in human THP-1 macrophages have identified the lipid metabolism-related proteins cluster of differentiation 36 (CD36) and perilipin-2 (PLIN2) as further regulatory targets of α-13′-COOH [8,12]. While Wallert et al. revealed that α-13′-COOH is involved in the modulation of foam cell formation at least in parts via an upregulation of CD36 expression [8], Schmölz et al. showed that α-13′-COOH is able to protect cells from stearic acid-induced lipotoxicity by upregulation of PLIN2 expression [12]. Interestingly, both studies also provided evidence that α-13′-COOH could be involved in the regulation of cellular neutral lipid accumulation by a hitherto-unknown mechanism independent of CD36 and PLIN2.

Cellular neutral lipid accumulation is predominantly driven by free-fatty acids (FFAs), which are obtained from chylomicrons and very low-density lipoproteins (VLDL) via enzymatic processing of their core triglycerides by lipoprotein lipase (LPL) on the cellular surface [13]. In vivo, LPL is produced in parenchymal cells and transported towards the vascular surface by the glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1). Here, catalytically-active LPL dimers are stabilized due the interaction with GPIHBP1, heparan sulfate proteoglycans (HSPGs) and substrate lipoproteins (extensively reviewed in [14]). However, in vitro studies have revealed that besides the normal production mechanism of LPL in vivo, macrophages are also able to produce and secrete functional LPL [15,16]. Due to its key role in cellular energy supply as well as the modulation of plasma triglyceride levels, LPL activity is under tight regulatory control. Several members of the family of angiopoietin-like (ANGPTL) proteins – with ANGPTL4 being the most prominent form in human macrophages – have emerged as potent physiological inhibitors of LPL activity [17,18]. Among others, the expression of ANGPTL4 is induced by FFAs via peroxisome proliferator-activated receptors (PPARs) as part of a feedback mechanism to prevent lipid overload within the cells [19,20].

There is growing evidence that elevated levels of triglyceride-rich lipoproteins represent causal risk factors for the development of cardiovascular diseases, making the LPL system and its associated regulator proteins interesting targets for investigations in that field [21,22]. Especially in macrophages, elevated accumulation of lipids due to the enhanced degradation of triglyceride-rich lipoproteins and the subsequent uptake of FFAs can promote foam cell formation and therefore the development of atherosclerosis [22,23]. In line with this, macrophage-derived foam cells have been identified as the primary source of LPL in human atherosclerotic plaques [15]. The present study will therefore focus on the potential regulation of LPL and ANGPTL4 by α-TOH and its LCM α-13′-COOH, as well as the impact of both compounds on VLDL-induced neutral lipid accumulation in human THP-1 macrophages as an important part of the development of lipid-driven diseases.

2. Materials and methods

2.1. Ethics statement

The collection of blood samples was conducted according to the principles expressed in the Declaration of Helsinki. The study protocol was approved by the ethical committee of the Friedrich Schiller University Jena (registration no. 2019-1548).

2.2. Chemicals

Unless indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Darmstadt, Germany), Thermo Fisher Scientific (Darmstadt, Germany), or Merck Millipore (Darmstadt, Germany).

2.3. Isolation of garcinoic acid and semi-synthesis of α-13′-COOH

The LCM α-13′-COOH was obtained via semi-synthesis from the natural compound garcinoic acid, also known as δ-tocotrienolic acid (δ-T3-13′-COOH). Garcinoic acid was isolated from the seeds of *Garcinia kola E. Heckel*, which were a kind gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid and subsequent synthesis of α-13′-COOH were performed as described in [10,24]. A purity of α-13′-COOH higher than 95% was confirmed by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

2.4. Determination of compound concentrations for cell culture studies

The concentrations of α-TOH and α-13′-COOH were determined weekly by absorption measurement in pure ethanol with a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The wavelengths and attenuation coefficients used were 292 nm and $\varepsilon = 3060$ for both compounds.

2.5. Cell culture

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultivated in Roswell Park Memorial Institute (RPMI)-1640 cell culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) Superior and 0.1% (v/v) penicillin-streptomycin-glutamine (PSG) solution. Cells were cultured at 37 °C in a humidified 5% CO_2/95% air atmosphere. Subsequent differentiation into macrophages was initiated by adding 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 50 μM β-mercaptoethanol for 96 h. After 96 h, fully matured macrophages were incubated with serum-free supplemented RPMI-1640 and the test compounds as indicated in the figures and were harvested for further processing as described below.

2.6. Incubation

α-Tocopherol, α-13′-COOH, orlistat and GSK3787 (Cayman Chemical, Hamburg, Germany) were dissolved in DMSO. For incubation of cells, compounds were mixed with serum-free supplemented RPMI-1640 medium in the concentrations indicated in the figures.

2.7. RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Qiagen, Venlo, Netherlands) including on-column DNase I (Qiagen) digestion as described in [25]. cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) and 500 ng/μl oligo-dT primers as described in [26].

2.8. Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) analyses were run on a LightCycler 480 II instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix as described in [26,27]. Primers for LPL, ANGPTL4 and RPL37A (reference gene) (Supplementary Table S1) were purchased from Thermo Fisher Scientific. PCR results were analyzed using the LightCycler software version 1.5.0.39 (Roche Diagnostics).

2.9. Immunoblotting

2.9.1. Sample preparation

2.9.1.1. Cell lysate. Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) containing 1% protease inhibitor and mixed 3:1 with SDS sample buffer (6.26% 1 M Tris-HCl, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue).

2.9.1.2. Cell culture supernatant. Protein precipitation was performed according to the protocol of Wessel and Flügge, with slight modifications [28]. A total volume of 700 µl cell culture supernatant was collected for each sample and mixed with 700 µl methanol as well as 175 µl chloroform by vortexing. Subsequent centrifugation at 13.000 ×g for 5 min using an Eppendorf 5417 R centrifuge (Eppendorf, Hamburg, Germany) resulted in the separation of three phases: (i) an upper aqueous/methanol phase, (ii) a protein layer, and (iii) a lower chloroform phase. The upper phase was carefully removed, and the remaining liquid was mixed with another 700 µl methanol by vortexing. After an additional centrifugation step at 13.000 ×g for 5 min, excess solvents were removed, and protein pellets were dried for 15 min at 55 °C. The samples were reconstituted in 80 µl 1× SDS sample buffer by resuspending and vortexing. After a short centrifugation, the samples were incubated at 70 °C for 5 min using an Eppendorf Themomixer comfort and subsequently placed on ice.

2.9.2. Western blotting

The proteins were separated by SDS-PAGE, using a 12% acrylamide gel and transferred to PVDF membrane (VWR, Darmstadt, Germany) using a transfer buffer containing 0.25 M Tris, 1.92 M glycine, 0.1% SDS and 20% methanol (pH 8.3). Primary antibodies against ANGPTL4 (rabbit anti-ANGPTL4; Abcam ab206420, Cambridge, UK; 1:500), LPL (rabbit anti-LPL; Abcam ab172953; 1:1000) and α-tubulin (mouse anti-α-tubulin clone B-5-1-2; BD Biosciences, Heidelberg, Germany; 1:5000) as well as secondary antibodies (swine anti-rabbit and rabbit anti-mouse labeled with horseradish peroxidase; DAKO, Hamburg, Germany; 1:5000) were used for investigating the proteins of interest. SignalBoost Immunoreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for enhancing chemiluminescence signals of ANGPTL4 and LPL antibodies, while the α-tubulin antibody was incubated in a hybridization buffer containing 0.5% milk powder and PBS (0.137 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4, pH 7.4).

2.9.3. Detection

For detection, Pierce ECL Western Blotting Substrate and CL-XPosure Films (Thermo Fisher Scientific) were applied. The exposure time for ANGPTL4 and LPL was 30 min, and for α-tubulin 1 min.

2.10. Isolation of very low-density lipoproteins

Very low-density lipoproteins were isolated from the blood of a fasted 28-year-old male and 31-year-old female with plasma triglyceride levels > 0.90 mmol/l, which was previously described as a suitable range for LPL assays [29,30]. For this, a total of 50 ml blood was collected in 9 ml EDTA-monovettes (Sarstedt, Nümbrecht, Deutschland). Blood samples were centrifuged at 1870 ×g for 10 min at 15 °C for plasma separation. Subsequently, plasma was transferred into 4 ml thickwall polycarbonate tubes (Beckmann Coulter, Brea, CA, USA) for ultracentrifugation. Ultracentrifugation was performed for 4 h at 15 °C and 269,200 ×g using an Optima LE 80 K ultracentrifuge (Beckmann Coulter) together with a Type 50.4 Ti rotor (Beckmann Coulter). Separated VLDL particles from both donors were pooled in 5 ml tubes (Eppendorf) and subsequently stored under nitrogen atmosphere at 4 °C. The protein concentration of the isolated VLDL was determined using Bio Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's operating instructions. Isolated VLDL was used within one week for the respective experimental procedures.

2.11. Measurement of lipoprotein lipase activity

Measurement of lipoprotein lipase activity was performed according to the procedures described in [30]. Human THP-1 monocytes were differentiated in a twelve-well cell culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) as described above, using 1×10^6 cells in 2 ml RPMI-1640 (plus 10% FBS and 0.1% PSG) per well. Subsequently, cell culture medium was removed and the fully matured macrophages were washed twice with PBS. Next, cells were pre-incubated according to the respective experimental design indicated in the figures in phenol red-free RPMI-1640 medium under serum-free conditions. Very low-density lipoproteins in a concentration equivalent to 50 µg/ml protein together with 0.5 µl quenched LPL substrate (Abcam ab214552) were added to the pre-incubated cells. The twelve-well plate was placed in the FLUOstar Omega microplate reader coupled to an atmospheric control unit (BMG Labtech), providing an atmosphere with 5% CO_2 at 37 °C. Fluorescence intensity (FI) of each well was determined hourly over 24 h at Ex/Em = 485/520 nm.

2.12. Flow cytometry to measure neutral lipids using Nile red

Measurement of cellular neutral lipid content was performed after completion of the LPL assay procedure. Cells were washed twice with PBS and detached by accutase treatment (30 min at 37 °C). Detached cells were collected in 2 ml tubes (Eppendorf), centrifuged (5 min, 400 ×g) and washed with PBS (this step was repeated twice). Next, cells were stained with 1 µg/ml Nile red solution, incubated for 10 min and washed again with PBS. Two additional wells with 1×10^6 cells (untreated and VLDL-treated) were included throughout the whole assay procedure to serve as unstained controls for Nile red staining. Flow cytometric analysis of neutral lipids was performed by measuring the emission at 570 to 590 nm using the Attune NxT Flow Cytometer (Thermo Fisher Scientific). Measurement results were analyzed using the Attune NxT software version 2.2.0.8543 (Thermo Fisher Scientific). A detailed description of the gating strategy used for generating flow cytometric data is provided in the Supplement.

2.13. Statistics

Data are presented as either means ± standard deviation (Figs. 3, 4, 5, 6, 7 B and C) or means ± standard error of the mean (Figs. 1, 2, and 7 A) of independent experiments as indicated. To test for statistical significance of the data shown in Figs. 1, 2, 3, 4, 6, 7 A and C, repeated measurement one-way Anova with Dunnett's post hoc test was performed using OriginPro software version 9.1G (OriginLab, Northampton, USA). To test for statistical significance of the data shown in Figs. 5 and 7 B, repeated measurement two-way Anova with Tukey's post hoc test was performed using SPSS software version 19.0 (IBM Deutschland GmbH, Ehningen, Germany). All experiments were performed in at least three independent biological replicates.

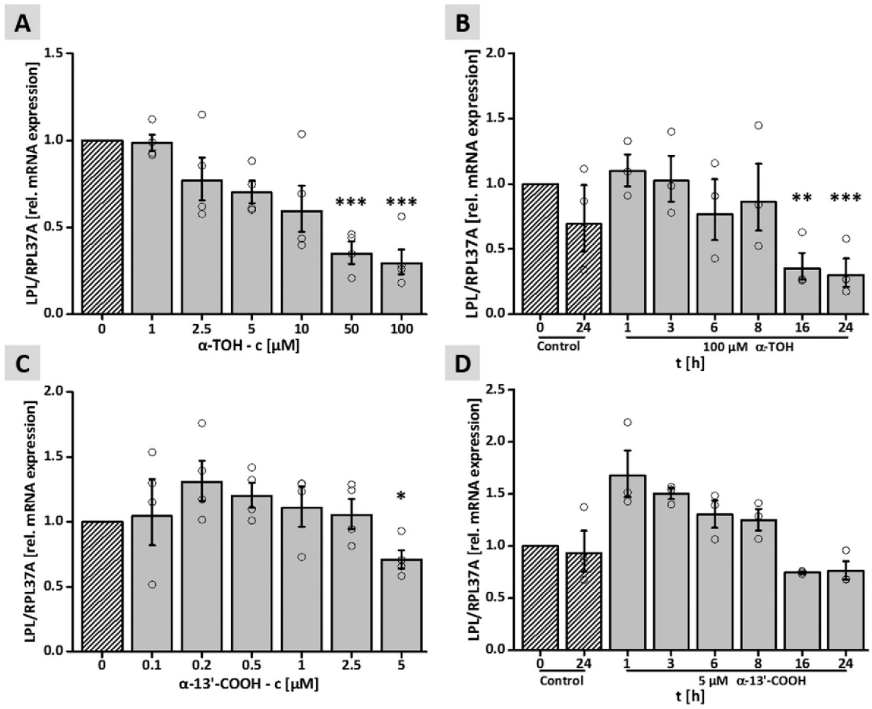

Fig. 1. LPL mRNA expression is reduced by α-TOH but not by α-13′-COOH. The influence of α-TOH and α-13′-COOH on LPL mRNA expression was investigated in a dose- and time-dependent manner under serum-free conditions. LPL mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Treatment with α-TOH in a concentration of 50 µM ($p < 0.001$) and 100 µM ($p < 0.001$) significantly reduced LPL mRNA expression, respectively. Lower α-TOH concentrations (2.5–10 µM) reduced LPL mRNA expression to a lower, non-significant extent (A). Time-dependent investigation of LPL mRNA expression confirmed the observed inhibitory effect of α-TOH at a concentration of 100 µM. Treatment with α-TOH significantly reduced LPL mRNA expression at the 16 h ($p < 0.01$) and 24 h ($p < 0.001$) time-points, respectively (B). In contrast to α-TOH, neither ascending concentrations of α-13′-COOH – aside from a small decrease with 5 µM – nor time-dependent investigation of the highest concentration used revealed significant effects of α-13′-COOH on LPL mRNA expression. Only marginal but non-significant induction of LPL mRNA expression was observed after a short time incubation with α-13′-COOH (1 and 3 h) at a concentration of 5 µM (C and D). Mean expression levels of four (A and C) or three (B and D) independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM). To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (A and C vs. control; B and D vs. 0 h control).

3. Results

The LCM α-13′-COOH emerged as a regulatory metabolite with specific effects that are more potent and even different to the effects of its vitamin precursor α-TOH [8–12,31]. Therefore, one of the main goals of the present study was to strengthen the new perspective on vitamin E metabolites as 'activated' or 'functional' metabolites by revealing another facet of their biological activity. It has recently been reported that α-13′-COOH is involved in regulating different aspects of the cellular lipid homeostasis – namely foam cell formation and lipotoxicity – via modulation of CD36 and PLIN2 expression [8,12]. In addition, there has also been evidence of the involvement of α-13′-COOH in regulating cellular neutral lipid accumulation by a hitherto-unknown mechanism independent of CD36 and PLIN2 (the reader is referred to section '1 Introduction'). Based on these results and the fact that lipid metabolism-related proteins represent regulatory targets of α-13′-COOH, the present study focused on the regulation of LPL and ANGPTL4 by α-TOH and its LCM α-13′-COOH. Furthermore, the impact of both compounds on VLDL-induced neutral lipid accumulation in human macrophages was investigated to extend the knowledge on foam cell formation from the previous works of Wallert et al. and Schmölz et al. [8,12].

3.1. LPL mRNA expression is reduced by α-TOH but not by α-13′-COOH

Lipoprotein lipase represents a key enzyme for the release of FFAs from triglyceride-rich lipoproteins and is therefore indirectly related to intracellular lipid storage [13]. Based on previous studies in THP-1 macrophages showing an impact of α-TOH and α-13′-COOH on neutral lipid accumulation, we investigated the effect of these compounds on LPL mRNA expression in a dose- and time-dependent manner by RT-qPCR (under serum-free conditions). First, human THP-1 macrophages were treated with ascending concentrations of either α-TOH (1–100 µM) or α-13′-COOH (0.1–5 µM) for 24 h. DMSO was used as the vehicle control (Fig. 1 A and C). In addition, THP-1 macrophages were harvested at different time points (1–24 h), using 100 µM α-TOH or 5 µM α-13′-COOH for incubation, respectively. DMSO vehicle control was prepared for the 0 h and 24 h time points (Fig. 1 B and D). Lipoprotein lipase mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown).

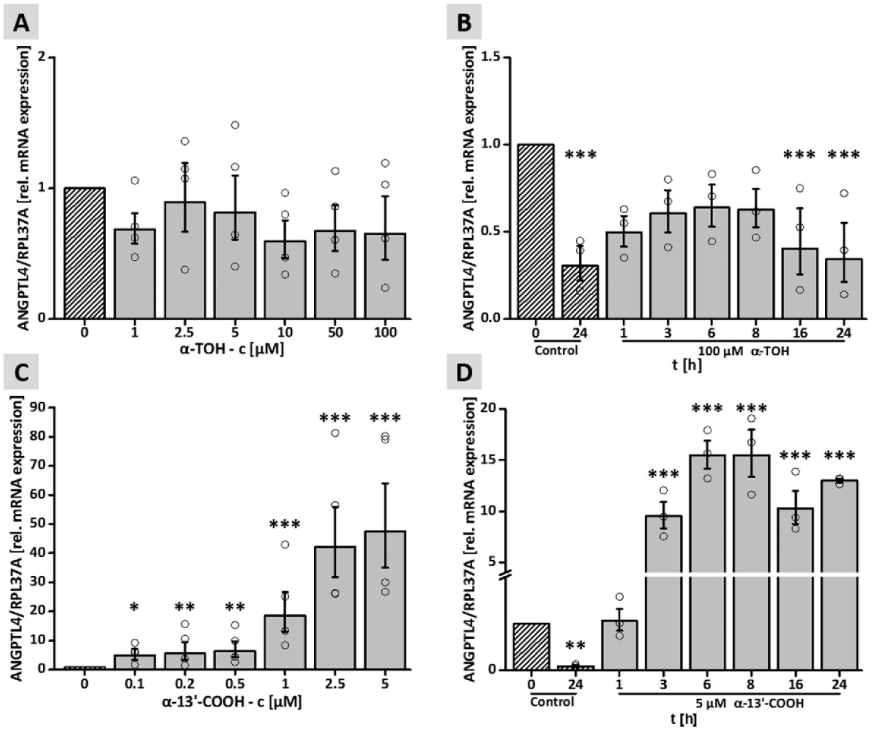

Fig. 2. ANGPTL4 mRNA expression is induced by α-13′-COOH but not by α-TOH. The influence of α-TOH and α-13′-COOH on ANGPTL4 mRNA expression was investigated in a dose- and time-dependent manner under serum-free conditions. ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Neither ascending concentrations nor time-dependent investigation of the highest concentration used revealed significant effects of α-TOH on ANGPTL4 mRNA expression (A and B). In tendency, α-TOH slightly reduced ANGPTL4 mRNA expression at all investigated concentrations, where the lowest (1 μM) and highest concentrations (100 μM) revealed nearly similar effects (A). Non-significant reduction of ANGPTL4 mRNA expression was also observed for time-dependent investigation after treatment with 100 μM α-TOH. Interestingly, ANGPTL4 mRNA expression was affected by serum-free conditions. Serum depletion for 24 h (24 h control) resulted in a significant reduction of ANGPTL4 mRNA expression ($p < 0.001$) compared to the 0 h control (B). In contrast to α-TOH, ascending concentrations of α-13′-COOH significantly enhanced ANGPTL4 mRNA expression in THP-1 macrophages ($p < 0.05$ to 0.001) (C). The effect of 5 μM α-13′-COOH was further investigated over different times. As already observed, 24 h serum depletion resulted in a significant reduction of ANGPTL4 mRNA expression compared with the 0 h control ($p < 0.01$). Nevertheless, treatment with 5 μM α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points – aside from 1 h ($p < 0.001$) (D). Mean expression levels of four (A and C) or three (B and D) independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM). To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (A and C vs. control; B and D vs. 0 h control).

Treatment with high concentrations of α-TOH (50 and 100 μM) significantly reduced LPL mRNA expression to 0.3-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.001$) and 0.3-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.001$), respectively. Lower concentrations of α-TOH (2.5–10 μM) had no significant effect on LPL mRNA expression (Fig. 1 A). Time-dependent investigation of LPL mRNA expression confirmed the observed inhibitory effect of α-TOH at a concentration of 100 μM. Treatment with α-TOH significantly reduced LPL mRNA expression at the 16 h and 24 h time points to 0.4-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.01$) and 0.4-fold (SEM min 0.01-fold, SEM max 0.1-fold, $p < 0.001$), respectively (Fig. 1 B). In contrast to α-TOH, neither ascending concentrations of α-13′-COOH – aside from a small decrease with 5 μM to 0.7-fold (SEM min 0.1-fold, SEM max 0.1-fold, $p < 0.05$) – nor time-dependent investigation of the highest concentration used revealed significant effects on LPL mRNA expression. Only a marginal but non-significant induction of LPL mRNA expression was observed after a short incubation period with α-13′-COOH (1 and 3 h) at a concentration of 5 μM (Fig. 1 C and D).

3.2. *ANGPTL4 mRNA expression is induced by α-13′-COOH but not by α-TOH*

Based on the initial results on LPL mRNA expression, we further explored the impact of both compounds on the expression of ANGPTL4, a potent physiological inhibitor of LPL activity. An upregulation of ANGPTL4 mRNA expression by α-TOH or α-13′-COOH could also represent an effective way of regulating cellular lipid storage [32,33]. Therefore, the influence of α-TOH and α-13′-COOH on ANGPTL4 mRNA expression was investigated in dose- and time-dependent manner by RT-qPCR (under serum-free conditions). First, human THP-1 macrophages were treated with ascending concentrations of either α-TOH (1–100 μM) or α-13′-COOH (0.1–5 μM) for 24 h. DMSO was used as vehicle control (Fig. 2 A and C). Next, THP-1 macrophages were harvested at different time points (1–24 h), using 100 μM α-TOH or 5 μM α-13′-COOH for

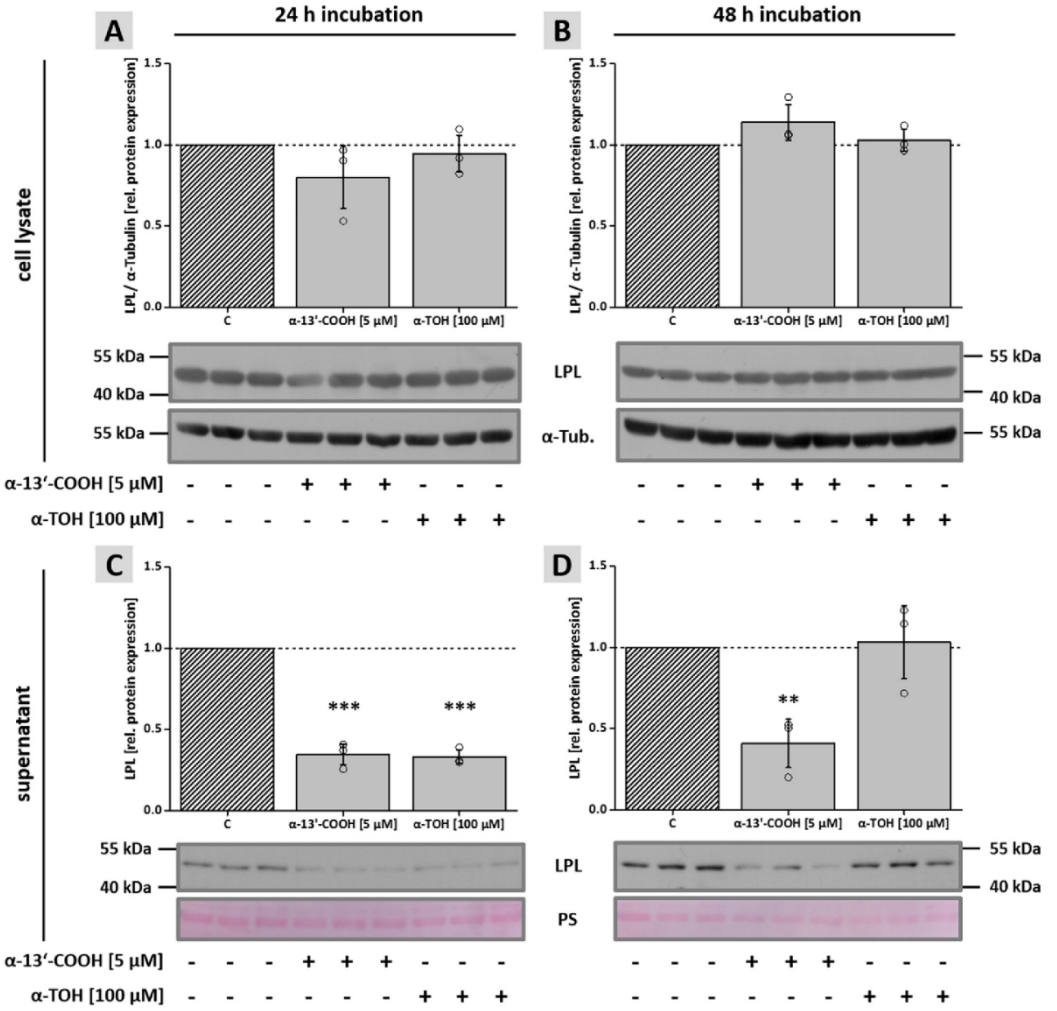

Fig. 3. The amount of secreted LPL protein is reduced by α-13′-COOH and α-TOH. Interestingly, expression of LPL protein (≈53 kDa) in the cell lysate was affected neither by α-TOH nor α-13′-COOH treatment after 24 h and 48 h incubation (A and B). However, Western blots of the corresponding cell culture supernatants revealed that both compounds affected the amount of secreted LPL protein (C and D). In line with the results from mRNA analysis, α-TOH treatment significantly reduced the secreted amount of LPL protein after 24 h compared with the vehicle control ($p < 0.001$) (C). After 48 h, the amount of secreted LPL was restored to the control level (D). Interestingly, α-13′-COOH also significantly reduced the secreted LPL amount after 24 h to the same extent as α-TOH compared with the vehicle control ($p < 0.001$) (C). In contrast to α-TOH, significant reduction of the secreted LPL amount was still present after 48 h in the α-13′-COOH-treated samples ($p < 0.01$) (D). Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control; lanes 4 to 6: 5 µM α-13′-COOH; lanes 7 to 9: 100 µM α-TOH). Mean relative protein expression levels of three independent biological experiments are shown (transparent points). Data is presented as means ± standard deviation. To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (vs. DMSO control). Abbreviations: α-Tub, α-tubulin; C, DMSO control; kDa, kilodalton; PS, Ponceau S staining.

incubation, respectively. DMSO vehicle control was prepared for the 0 h and 24 h time points (Fig. 2 B and D). ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). Neither ascending concentrations nor time-dependent investigation of the highest concentration used revealed significant effects of α-TOH on ANGPTL4 mRNA expression. In tendency, α-TOH slightly reduced ANGPTL4 mRNA expression at all investigated concentrations, with the lowest (1 µM) and the highest concentration (100 µM) revealing nearly similar effects. Non-significant reduction of ANGPTL4 mRNA expression was also observed for time-dependent investigation after treatment with 100 µM α-TOH, aside from the late time points (16 h and 24 h). Interestingly, ANGPTL4 mRNA expression was affected by serum depletion. Serum-free conditions for 24 h (24 h control) resulted in a reduction of

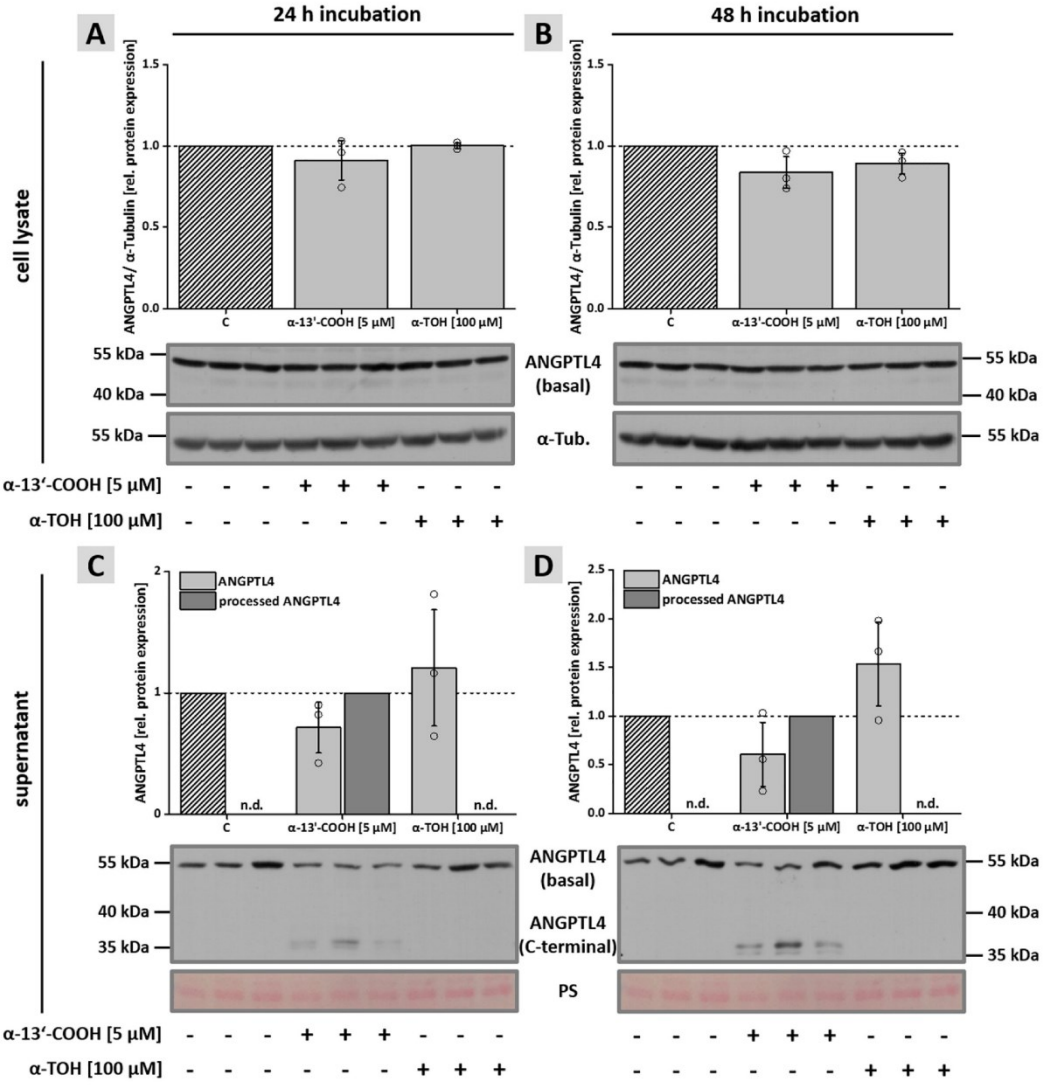

Fig. 4. Processing of ANGPTL4 protein is induced by α-13′-COOH but not by α-TOH. In line with the results from mRNA analysis, α-TOH treatment did not significantly affect ANGPTL4 (≈50 kDa) expression in cell lysates as well as secretion of ANGPTL4 protein to cell culture supernatant compared with the vehicle control (A to D). Surprisingly, α-13′-COOH also had no effect on ANGPTL4 protein expression in cell lysates after 24 h and 48 h incubation (A and B). Furthermore, Western blots of the corresponding cell culture supernatants revealed that α-13′-COOH slightly, but not significantly reduced the secretion of basal ANGPTL4 protein after 24 h and 48 h incubation (C and D). However, an additional band representing the C-terminal fragment of ANGPTL4 (≈37 kDa) could be exclusively detected in supernatants of α-13′-COOH treated samples. The amount of secreted C-terminal fragments further increased after 48 h incubation (C and D). Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control; lanes 4 to 6: 5 μM α-13′-COOH; lanes 7 to 9: 100 μM α-TOH). Mean relative protein expression levels of three independent biological experiments are shown (transparent points). Data is presented as means + standard deviation. To test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used (vs. DMSO control A and D, C and D only for basal ANGPTL4). For quantifying the amount of C-terminal ANGPTL4 cleavage product, α-13′-COOH treatment and not DMSO control was used as reference (C and D, processed ANGPTL4). For this, the relative amount of C-terminal ANGPTL4 in the α-13′-COOH treated samples were set to one as a fixed value, while the absence of C-terminal ANGPTL4 in DMSO control and α-TOH treated samples were marked as not detectable (n.d.). Because of the missing values for C-terminal ANGPTL4 in DMSO control and α-TOH treated samples, no calculation of statistical significance was possible (C and D, processed ANGPTL4). Abbreviations: α-Tub, α-tubulin; C, DMSO control; kDa, kilodalton; n.d., not detectable; PS, Ponceau S staining.

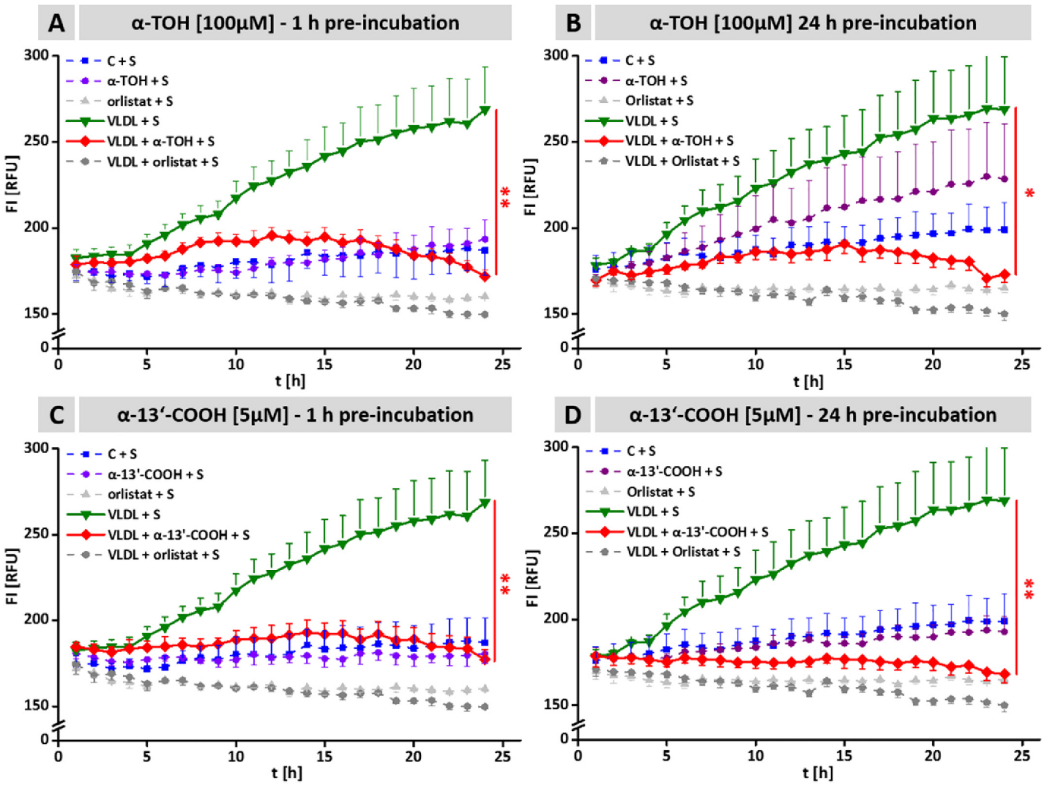

Fig. 5. Lipoprotein lipase activity is reduced by α-13′-COOH and α-TOH. Both α-TOH (100 µM) and α-13′-COOH (5 µM) appeared as potent inhibitors of VLDL-induced LPL activity. Already after 1 h pre-incubation with the two compounds, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH or α-13′-COOH evolved in a significantly different range ($p < 0.01$) (A and C). Elongation of the pre-incubation time with α-TOH or α-13′-COOH to 24 h revealed similar results. Again, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH ($p < 0.05$) or α-13′-COOH ($p < 0.01$) evolved in a significantly different range (B and D). As expected, orlistat (negative control) significantly blocked LPL activity compared with VLDL treatment and the vehicle control (significance not shown). Mean FI values of four independent biological experiments are shown. Data is presented as means ± standard deviation. In order to test for statistical significance, a repeated measurement two-way Anova with Tukey post hoc test was used. *, $p < 0.05$; **, $p < 0.01$ (vs. VLDL). Abbreviations: C, untreated control; FI, fluorescence intensity; RFU, relative fluorescence units; S, fluorescence-labeled LPL substrate.

ANGPTL4 mRNA expression to 0.4-fold (SEM min 0.1-fold, SEM max 0.2-fold, $p < 0.001$) compared to 0 h control, which almost corresponds to the values of 16 h and 24 h α-TOH treatment. Therefore, the observed time-dependent reduction of ANGPTL4 mRNA expression is probably not attributed to α-TOH but rather to serum depletion (Fig. 2 A and B). In contrast to α-TOH, ascending concentrations of α-13′-COOH significantly increased ANGPTL4 mRNA expression in THP-1 macrophages. Already 0.1 µM α-13′-COOH enhanced ANGPTL4 mRNA expression to 4.9-fold (SEM min 1.5-fold, SEM max 2.2-fold, $p < 0.05$), while treatment with 5 µM increased ANGPTL4 mRNA expression to 47.5-fold (SEM min 12.2-fold, SEM max 16.6-fold, $p < 0.001$) (Fig. 2 C). The effect of 5 µM α-13′-COOH was further investigated at different time points. As already observed, 24 h serum depletion resulted in a significant reduction of ANGPTL4 mRNA expression to 0.1-fold (SEM min 0.2-fold, SEM max 0.2-fold, $p < 0.001$) compared with the 0 h control. Nevertheless, treatment with 5 µM α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points – aside from 1 h – between 9.5-fold and 15.5-fold ($p < 0.001$). At the 24 h time point, α-13′-COOH treatment increased ANGPTL4 mRNA expression to 13.0-fold (SEM min 0.2-fold, SEM max 0.2-fold, $p < 0.001$). Time-dependent experiments showed a substantially lower increase of ANGPTL4 mRNA expression than dose-dependent experiments, indicating that the high induction of ANGPTL4 in dose-dependent experiments is partially the result of the 24 h serum depletion (Fig. 2 D).

3.3. *The amount of secreted LPL protein is reduced by α-TOH and α-13′-COOH*

In order to examine whether the observed changes in LPL mRNA expression are also present at the protein level, we performed Western blot analyses of cell lysates and cell culture supernatants of human THP-1 macrophages. Cell culture supernatants were analyzed because LPL protein is secreted in its monomeric form and subsequently anchored on the cellular surface by the interaction with GPIHBP1 and HSPGs. Here, LPL is converted to its catalytically-active dimeric form [14]. Hence, the reduced secretion of LPL monomers by α-TOH or α-13′-COOH treatment could also result in a reduction of LPL activity. In order to investigate LPL protein, human THP-1 macrophages were treated with 5 µM α-13′-

COOH or 100 μM α-TOH for 24 h or 48 h under serum-free conditions. DMSO was used as vehicle control. Each of the presented Western blot images comprises three independent biological replicates (lanes 1 to 3: DMSO control, lanes 4 to 6: 5 μM α-13′-COOH, and lanes 7 to 9: 100 μM α-TOH). α-Tubulin and Ponceau S staining were used as loading controls for cell lysates and cell culture supernatants, respectively. Expression of LPL was detected using a recombinant antibody (Abcam ab172953) against the C-terminal residue of the protein. Interestingly, the expression of LPL protein (≈53 kDa) in the cell lysate was affected neither by α-TOH nor α-13′-COOH treatment after 24 h and 48 h incubation (Fig. 3 A and B). However, Western blots of the corresponding cell culture supernatants revealed that both compounds affected the amounts of secreted LPL protein. In line with the results from mRNA analyses, α-TOH treatment significantly reduced the secreted amount of LPL protein after 24 h to 0.33-fold ± 0.04-fold ($p < 0.001$) compared with the vehicle control (Fig. 3 C). After 48 h, the secreted amount of LPL protein was restored to the control level (Fig. 3 D). Surprisingly, α-13′-COOH also significantly reduced the secreted LPL amount after 24 h to the same extent as α-TOH (to 0.35-fold ± 0.06-fold, $p < 0.001$) compared with the vehicle control (Fig. 3 C). In contrast to α-TOH, the significant reduction of secreted LPL protein was still present after 48 h in the α-13′-COOH-treated samples (to 0.41-fold ± 0.15-fold, $p < 0.01$) (Fig. 3 D).

3.4. Processing of ANGPTL4 protein is induced by α-13′-COOH but not by α-TOH

Based on the observed induction of ANGPTL4 mRNA expression, we were interested in exploring whether the unexpected reduction of LPL protein secretion by α-13′-COOH could be explained by an ANGPTL4-dependent mechanism. The ANGPTL4 protein (≈50 kDa) comprises a C-terminal (≈37 kDa) and an N-terminal domain (≈15 kDa) with distinct functions. The N-terminal domain is responsible for the inhibition of LPL activity due to the conversion of catalytically-active LPL to the inactive form [34], while the C-terminal domain mediates anti-angiogenic functions [35]. Both domains are connected by a linker, which can be cleaved by pro-protein convertases subtilisin/kexin (PCSKs) [36]. In order to investigate whether ANGPTL4 expression and cleavage is affected by α-TOH or α-13′-COOH, we performed Western blot analyses of THP-1 cell lysates as well as cell culture supernatants. Cell culture supernatants were studied because ANGPTL4 is also a secreted protein that is expected to follow the similar route to the cell surface as LPL [37]. To ensure specific detection of the ANGPTL4 protein, we had to use a recombinant antibody (Abcam, ab206420) against the C-terminal region of the protein, although the N-terminal fragment would be of greater interest for the inhibition of LPL activity. Unfortunately, no specific recombinant antibody against the N-terminal fragment was commercially available. However, previous studies have shown that if the C-terminal fragment (≈37 kDa) can be detected, the N-terminal fragment (≈15 kDa) is also present [38]. For the investigation of ANGPTL4 protein, human THP-1 macrophages were treated with 5 μM α-13′-COOH or 100 μM α-TOH for 24 h or 48 h under serum-free conditions. DMSO was used as vehicle control. Each of the presented Western blot images comprises three independent biological replicates (lanes 1–3 DMSO control, lanes 4–6 5 μM α-13′-COOH, and lanes 7–9 100 μM α-TOH). α-Tubulin and Ponceau S staining were used as loading controls for cell lysate and cell culture supernatant, respectively.

In line with the results from mRNA analysis, α-TOH treatment did not significantly affect ANGPTL4 (≈50 kDa) expression in cell lysates as well as secretion of ANGPTL4 protein to cell culture supernatant compared with the vehicle control (Fig. 4 A to D). Surprisingly, α-13′-COOH also had no effect on ANGPTL4 protein expression in cell lysates after 24 h and 48 h incubation (Fig. 4 A and B). Furthermore, Western blots of the corresponding cell culture supernatants revealed that α-13′-COOH slightly, but not significantly reduced the secretion of basal ANGPTL4 protein after 24 h (to 0.71-fold ± 0.21-fold) and 48 h (to 0.61-fold ± 0.33-fold) incubation (Fig. 4 C and D). However, the most interesting observation was the appearance of an additional band, representing the C-terminal fragment of ANGPTL4 (≈37 kDa), exclusively in the supernatants of α-13′-COOH treated samples. The amount of C-terminal ANGPTL4 further increased with 48 h incubation time (Fig. 4 C and D). In summary, while α-13′-COOH-triggered induction of ANGPTL4 mRNA expression did not affect the expression of full-length ANGPTL4, the amount of the secreted processed C-terminal form was increased.

3.5. Lipoprotein lipase activity is reduced by α-TOH and α-13′-COOH

Based on the results from mRNA and protein investigations, α-TOH and α-13′-COOH seem to affect the LPL system through different mechanisms. The catalytic activity of LPL is under tight regulatory control at the transcriptional and post-translational level, among others by ANGPTL4 [32,39]. The function of LPL is predominantly considered to be anti-atherogenic [40]. However, LPL has also been associated with pro-atherogenic effects [41]. Especially in macrophages, enhanced degradation of triglyceride-rich lipoproteins and subsequent uptake of FFAs represents a risk factor for foam cell formation [17,22,23]. We were therefore interested in ascertaining whether α-TOH and α-13′-COOH affect the catalytic activity of LPL in human THP-1 macrophages. In our macrophage model, VLDL isolated from human donors was used as a physiological ligand for the stimulation of LPL activity. Furthermore, orlistat – a well-established and clinically-used LPL inhibitor – was included as a negative control [42]. In the experimental procedure, human THP-1 macrophages were pre-incubated with α-TOH (100 μM), α-13′-COOH (5 μM) or orlistat (50 μM, negative control) for 1 h (Fig. 5 A and C) or 24 h (Fig. 5 B and D) under serum-free conditions to investigate the effect of short- and long-term exposure to the two compounds. DMSO was used as vehicle control. Subsequently, VLDL in a concentration equivalent to 50 μg/ml protein together with 0.5 μl quenched, fluorescence-labeled LPL substrate was added to the pre-incubated cells. Analysis of LPL activity was carried out by hourly determination of fluorescence intensity as an equivalent for the processed substrate over 24 h.

Both α-TOH (100 μM) and α-13′-COOH (5 μM) appeared as potent inhibitors of VLDL-induced LPL activity. Already after 1 h pre-incubation with the two substances, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH or α-13′-COOH evolved in a significantly different range ($p < 0.01$) (Fig. 5 A and C). For α-TOH, the highest reduction of FI values was determined after 24 h from 268.8 ± 24.7 relative fluorescence units (RFU) in the VLDL-treated sample compared with 172.0 ± 3.7 RFU in the combination of α-TOH and VLDL (Fig. 5 A). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 268.8 ± 24.7 RFU in the VLDL-treated sample compared with 177.2 ± 5.8 RFU in the combination of α-13′-COOH and VLDL (Fig. 5 C). Elongation of the pre-incubation time with α-TOH or α-13′-COOH to 24 h revealed similar results. Again, FI values of the VLDL-treated sample (positive control) and the combination with either α-TOH ($p < 0.05$) or α-13′-COOH ($p < 0.01$) evolved in a significantly different range (Fig. 5 B and D). For α-TOH, the highest reduction of FI values was determined after 24 h from 269.0 ± 30.3 RFU in the VLDL-treated sample compared with 173.0 ± 4.7 RFU in the combination of α-TOH and VLDL (Fig. 5 B). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 269.0 ± 30.3 RFU in the VLDL-treated sample compared with 168.3 ± 5.2 RFU in the combination of α-13′-COOH and VLDL (Fig. 5 D). As expected, orlistat (negative control) significantly blocked LPL activity compared with VLDL treatment and the vehicle control (significance not shown). These results show that α-TOH and α-13′-COOH both inhibit the catalytic activity of LPL in human THP-1 macrophages.

3.6. VLDL-induced neutral lipid accumulation is attenuated by α-13′-COOH but not by α-TOH

It is well established that VLDL promotes the accumulation of neutral lipids in macrophages [43–45]. Furthermore, enhanced VLDL levels have been associated with pro-atherogenic effects like foam cell formation [22,46]. Based on the observed inhibition of LPL activity by α-TOH and α-13′-COOH, we were interested in ascertaining whether both compounds could attenuate neutral lipid accumulation in the presence of VLDL oversupply. Because the determination of neutral lipid accumulation was accomplished in the same sample, human THP-1 macrophages were treated as described in the experimental procedure for measuring LPL activity. Analyses were carried out by flow cytometry after Nile red staining. As expected, VLDL treatment significantly increased neutral lipid accumulation in human THP-1 macrophages compared with the vehicle control ($p < 0.001$). After 1 h pre-incubation with α-TOH (100 μM) or α-13′-COOH (5 μM), both compounds were unable to reduce VLDL-induced neutral lipid accumulation. Interestingly, co-incubation with α-TOH even increased the accumulation of neutral lipids to 114% ± 10% compared with VLDL alone (100%, $p < 0.01$) and with the combination of VLDL and α-13′-COOH (101% ± 5%, $p < 0.05$) (Fig. 6 A). After 24 h pre-incubation, VLDL-induced neutral

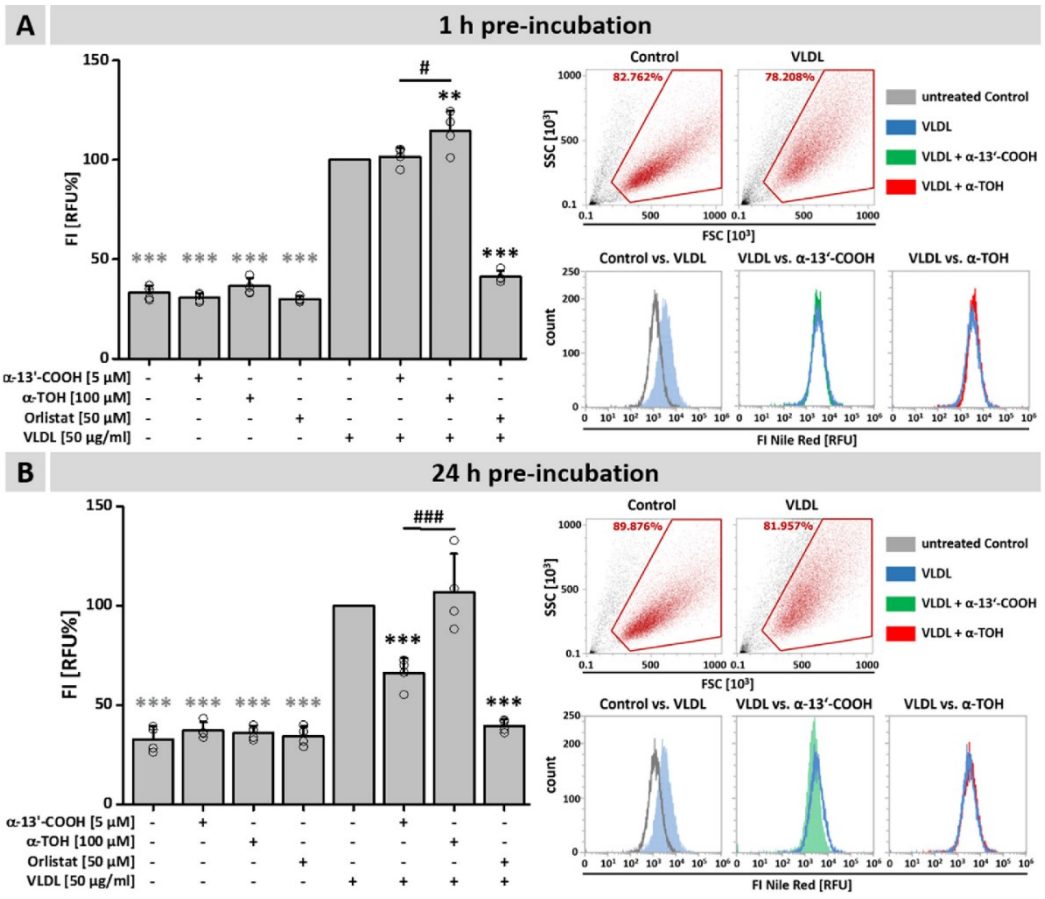

Fig. 6. VLDL-induced neutral lipid accumulation is attenuated by α-13′-COOH but not by α-TOH. As expected, VLDL treatment significantly increased neutral lipid accumulation in human THP-1 macrophages compared with the vehicle control ($p < 0.001$) (A and B). After 1 h pre-incubation, neither co-incubation with α-TOH (100 μM) nor α-13′-COOH (5 μM) reduced VLDL-induced neutral lipid accumulation. Interestingly, co-incubation with α-TOH even increased the accumulation of neutral lipids compared with VLDL alone ($p < 0.01$) and with the combination of VLDL and α-13′-COOH ($p < 0.05$) (A). After 24 h pre-incubation, VLDL-induced neutral lipid accumulation was significantly reduced by co-incubation with α-13′-COOH ($p < 0.001$). Interestingly, co-incubation with α-TOH again slightly increased neutral lipid accumulation compared with VLDL alone. There was also a significant difference between the effects of α-TOH and α-13′-COOH ($p < 0.001$) (B). As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages after 1 h and 24 h pre-incubation time ($p < 0.001$) (A and B). Mean FI values [%] of four independent biological experiments are shown (transparent points). Data is presented as means ± standard deviation. In order to test for statistical significance, a repeated measurement one-way Anova with Dunnett's post hoc test was used. **, $p < 0.01$; ***, $p < 0.001$ (vs. control), #, $p < 0.05$, ###, $p < 0.001$ (vs. α-TOH). The cell population of interest was identified using a gating strategy based on forward (FSC) and sideward scatter (SSC) data. Abbreviations: RFU, relative fluorescence units.

lipid accumulation was significantly reduced by co-incubation with α-13′-COOH to 66.0% ± 7.6% ($p < 0.001$). Surprisingly, co-incubation with α-TOH did not reduce neutral lipid accumulation, contrasting the expectation based on the LPL activity data. Again, co-incubation with α-TOH slightly increased neutral lipid accumulation to 106% ± 19% compared with VLDL alone. There was also a significant difference between the effect sizes of α-13′-COOH compared with α-TOH ($p < 0.001$) (Fig. 6 B). As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages after 1 h (41% ± 3%) and 24 h (40% ± 3%) pre-incubation time ($p < 0.001$) (Fig. 6 A and B). Taken together, VLDL-induced neutral lipid accumulation in THP-1 macrophages was attenuated by 24 h pre-incubation with α-13′-COOH, while α-TOH showed no effect.

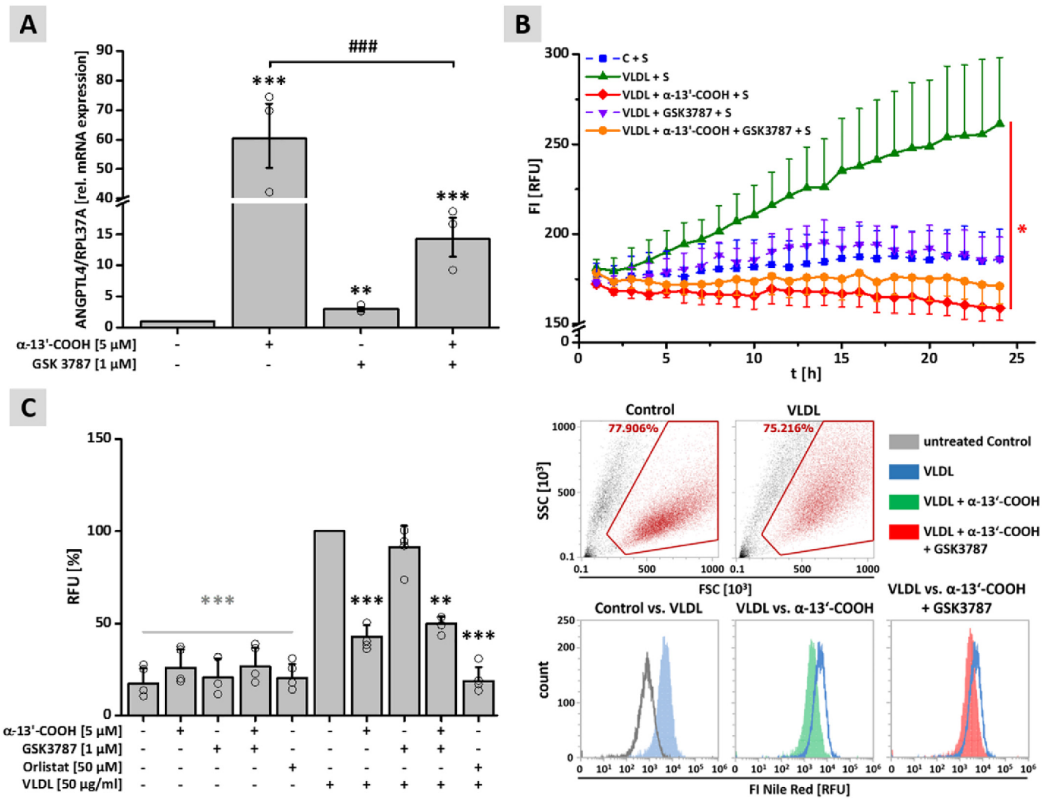

Fig. 7. PPARδ is involved in the transcriptional regulation of ANGPTL4 but does not affect the α-13′-COOH-mediated inhibition of LPL activity and neutral lipid accumulation. ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown) (A). As expected from our previous results, α-13′-COOH significantly increased ANGPTL4 mRNA expression ($p < 0.001$) compared with the vehicle control. However, the combination of α-13′-COOH and the PPARδ antagonist GSK3787 attenuated this effect, resulting in a significantly lower induction of ANGPTL4 mRNA expression ($p < 0.001$) compared with the vehicle control. Interestingly, the PPARδ antagonist GSK3787 alone also increased ANGPTL4 mRNA expression ($p < 0.01$) compared with the vehicle control (A).

Analysis of LPL activity was carried out by hourly determination of fluorescence intensity over 24 h (B), with subsequent analysis of neutral lipid accumulation via flow cytometry (C). As expected from our previous results, FI values of the VLDL-treated sample (positive control) and the combination with α-13′-COOH evolved in a significantly different range ($p < 0.05$). However, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on VLDL-induced LPL activity. For better clarity, VLDL-free treatments as well as orlistat negative controls are not shown. However, orlistat significantly blocked LPL activity compared with VLDL treatment and the vehicle control ($p < 0.001$) (B). As already observed, co-incubation with α-13′-COOH significantly reduced VLDL-induced neutral lipid accumulation ($p < 0.001$). However, in line with the results from the determination of LPL activity, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on VLDL-induced triglyceride accumulation. There was still a significant reduction ($p < 0.01$) in the combination of α-13′-COOH, GSK3787 and VLDL compared with the VLDL-treated samples. As expected, orlistat (negative control) significantly blocked neutral lipid accumulation in human THP-1 macrophages (C). The cell population of interest was identified using a gating strategy based on forward (FSC) and sideward scatter (SSC) data.

Mean expression levels of three independent biological experiments are shown (transparent points). Data is presented as means ± standard error of the mean (SEM) (A). Mean FI values of four independent biological experiments are shown (B). Mean FI values [%] of four independent biological experiments are shown (transparent points) (C). Data is presented as means ± standard deviation (B and C). In order to test for statistical significance, either a repeated measurement one-way Anova with Dunnett's post hoc test was performed using OriginPro 9.1G software (A and C) or a repeated measurement two-way Anova with Tukey post hoc test was performed using SPSS 19.0 software (B). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs. control (A) or vs. VLDL (B and C)), ###, $p < 0.001$ (vs. α-13′-COOH (A)). Abbreviations: C, untreated control; FI, Fluorescence intensity; RFU, relative fluorescence units; S, fluorescence-labeled LPL substrate.

3.7. PPARδ is involved in the transcriptional regulation of ANGPTL4 but does not affect α-13′-COOH-mediated inhibition of LPL activity and neutral lipid accumulation

Due to the promising effects of α-13′-COOH in preventing excessive lipid accumulation in human THP-1 macrophages, we were further interested in ascertaining the signaling mechanism underlying these effects. We first investigated the transcriptional regulation of ANGPTL4, based on the strong induction of ANGPTL4 mRNA expression in response to α-13′-COOH treatment. It is well-established that transcriptional regulation of ANGPTL4 is predominantly mediated by PPARs [17,47–49], with PPARδ representing the predominant PPAR form in human THP-1 macrophages [50]. We were therefore interested in determining whether the stimulation of PPARδ by α-13′-COOH could be responsible for the observed induction of ANGPTL4 mRNA expression as well as the inhibition of LPL activity and triglyceride accumulation. For mRNA investigations, human THP-1 macrophages were pre-incubated with GSK3787 (1 μM) – a specific and potent antagonist for PPARδ [51] – for 1 h under serum-free conditions. Subsequently, α-13′-COOH (5 μM) was added to the pre-incubated cells for another 24 h. DMSO was used as vehicle control (Fig. 7 A). As expected from our previous results, α-13′-COOH significantly increased ANGPTL4 mRNA expression to 60.0-fold (SEM min 9.9-fold, SEM max 11.9-fold) ($p < 0.001$) compared with the vehicle control. However, the combination of α-13′-COOH and GSK3787 attenuated this effect, resulting in a lower induction of ANGPTL4 mRNA expression to only 14.2-fold (SEM min 2.8-fold, SEM max 3.5-fold) ($p < 0.001$) compared with the vehicle control. The difference of the effect sizes between α-13′-COOH alone and in combination with GSK3787 was also significant ($p < 0.001$). Interestingly, the PPARδ antagonist GSK3787 alone also increased ANGPTL4 mRNA expression to 2.9-fold (SEM min 0.3-fold, SEM max 0.34-fold) ($p < 0.01$) compared with the vehicle control. Hence, PPARδ seems to be at least partly involved in the α-13′-COOH-dependent induction of ANGPTL4 mRNA expression (Fig. 7 A).

Based on this promising result, we were further interested in whether co-incubation with GSK3787 could also reduce the α-13′-COOH mediated effects on LPL activity and neutral lipid accumulation. Therefore, human THP-1 macrophages were treated as described for the measurement of LPL activity with slight modifications. The α-TOH treatment was replaced by GSK3787. Furthermore, two additional samples with co-incubation of α-13′-COOH and GSK3787 were included in the experimental setup. These samples were pre-incubated with GSK3787 for 1 h and subsequently treated with α-13′-COOH for an additional 24 h. Analysis of LPL activity was carried out by hourly determination of FI over 24 h (Fig. 7 B), with subsequent analysis of neutral lipid accumulation via flow cytometry (Fig. 7 C). As expected from our previous results, FI values of the VLDL-treated sample (positive control) and the combination with α-13′-COOH evolved in a significantly different range ($p < 0.05$). For α-13′-COOH, the highest reduction of FI values was determined after 24 h from 261.5 ± 36.8 RFU in the VLDL-treated sample compared with 158.8 ± 6.9 RFU in the combination of α-13′-COOH and VLDL (Fig. 7 B). However, co-incubation of α-13′-COOH and GSK3787 did not attenuate the inhibitory effect of α-13′-COOH on LPL activity. There was still a reduction of the FI values from 261.5 ± 36.8 RFU in the VLDL-treated sample compared with 171.0 ± 9.1 RFU in the combination of α-13′-COOH, GSK3787 and VLDL. However, orlistat significantly blocked LPL activity compared with VLDL treatment and the vehicle control ($p < 0.001$). For better clarity, VLDL-free treatments as well as orlistat negative controls are not shown (Fig. 7 B). As already observed, co-incubation with α-13′-COOH significantly reduced VLDL-induced neutral lipid accumulation to 42.8% ± 6.2% ($p < 0.001$). In line with the results from the determination of LPL activity, co-incubation of α-13′-COOH and GSK3787 was unable to attenuate the inhibitory effect of α-13′-COOH on VLDL-induced neutral lipid accumulation. There was still a significant reduction to 49.7% ± 4.1% ($p < 0.01$) in the combination of α-13′-COOH, GSK3787 and VLDL compared with the VLDL-treated samples. As expected, orlistat significantly blocked neutral lipid accumulation in human THP-1 macrophages (18.6% ± 7.5%) (Fig. 7 C). Taken together, the induction of ANGPTL4 mRNA expression by α-13′-COOH is diminished by pre-incubation with the PPARδ antagonist GSK3787. However, GSK3787 failed to attenuate the inhibitory effects of α-13′-COOH on LPL activity and neutral lipid accumulation.

4. Discussion

In a recently-postulated hypothesis on the significance of vitamin E metabolism in humans, LCMs were suggested as activated and therefore functional molecules of their vitamin precursors that must be taken into consideration for a correct interpretation of physiological effects of vitamin E in humans [2]. The concept of a central metabolic activation in the liver and the subsequent distribution of functional metabolites in the body is supported by the presence of α-13′-COOH in human serum [8]. Therefore, α-13′-COOH could theoretically be provided to various sites of action, including intimal macrophages, an important factor in the development of atherosclerosis. Two previous studies have already revealed an involvement of α-13′-COOH in the regulation of foam cell formation at least in part via the modulation of CD36 and PLIN2 expression [8,12]. However, there is also evidence of further mechanisms independent of CD36 and PLIN2. Using the above-mentioned investigations as a starting point, our aim was to elucidate the regulatory potential of α-13′-COOH and its vitamin precursor α-TOH on VLDL-mediated foam cell formation, focusing on the regulation of LPL and ANGPTL4.

As the regulatory potential of α-TOH and α-13′-COOH on lipid metabolism-related genes has already been shown in previous studies, we initially investigated the effect of both compounds on LPL and ANGPTL4 mRNA expression. Interestingly, the two compounds revealed different effects on the investigated genes. While α-TOH reduced LPL mRNA expression, it had no effect on the expression of ANGPTL4 (Figs. 1 and 2). By contrast, α-13′-COOH treatment strongly enhanced ANGPTL4 mRNA expression and did not affect LPL (Figs. 1 and 2). To the best of our knowledge, we are the first to describe an inhibition of LPL mRNA expression by α-TOH as well as the induction of ANGPTL4 mRNA expression by its LCM α-13′-COOH. Interestingly, ANGPTL4 mRNA expression was also affected by serum-free conditions. Especially in the α-TOH treated samples, the observed time-dependent reduction of ANGPTL4 mRNA expression is probably not attributed to compound treatment but rather to serum depletion. However, treatment with α-13′-COOH significantly enhanced ANGPTL4 mRNA expression at all investigated time points, although the effect of serum depletion was present. Since α-TOH and α-13′-COOH revealed their strongest effects on the mRNA level at a non-toxic concentration of 100 μM or 5 μM, respectively, we decided to use these concentrations for further experiments. In addition, identical concentrations of both compounds have also been used in other studies [8,10–12,31].

Observations at the protein level revealed a more complex picture. Since LPL and ANGPTL4 are both secreted proteins, occurrence in cell lysates and cell culture supernatants was investigated. Surprisingly, the protein expression of LPL was not affected by α-TOH in cell lysates, although it would have been expected from the previous mRNA results. However, Western blots of the corresponding supernatant showed that α-TOH reduced the secretion of LPL monomers after 24 h pre-incubation (Fig. 3). Previous studies – especially in adipocytes – have shown that the modulation of LPL mRNA expression also results in changes of LPL protein synthesis or activity [52,53]. We therefore conclude that the reduced amount of LPL monomers in cell culture supernatant after 24 h α-TOH treatment probably resulted from diminished LPL mRNA expression. We further conclude that α-TOH mediates its inhibitory effect on LPL protein expression via direct transcriptional regulation, as the compound did not affect ANGPTL4 mRNA and protein expression (Fig. 4). In line with our mRNA results, α-13′-COOH treatment did not

affect LPL expression in cell lysates. However, the amount of LPL monomers in the cell culture supernatant diminished after 24 h and 48 h (Fig. 3). Intracellular expression of full-length ANGPTL4 (≈50 kDa) was also not affected by α-13′-COOH treatment, although it would have been expected from mRNA results. Interestingly, Western blots of the corresponding cell culture supernatants revealed an enhanced amount of C-terminal ANGPTL4 (≈37 kDa) (Fig. 4). It should be noted that we had to use a recombinant antibody against the C-terminal region of the protein to ensure specific detection. Unfortunately, we were unable to find a commercial antibody for the specific and reliable detection of the N-terminal ANGPTL4 fragment, although this fragment would have been of greater interest for LPL inhibition. However, it was shown that if the C-terminal fragment is detectable, the N-terminal fragment (≈15 kDa) is also present [38]. We conclude that the induction of ANGPTL4 mRNA expression by α-13′-COOH results in a subsequent increase of protein expression, although basal levels of full-length ANGPTL4 protein remained unchanged. The increase of ANGPTL4 protein amount is probably masked by enhanced processing and the subsequent secretion of cleavage products (C-terminal and the N-terminal fragments), indicated by the detection of C-terminal ANGPTL4 fragments in the supernatant of α-13′-COOH-treated samples. Makoveichuk et al. reported similar results in a study in human THP-1 macrophages. Here, treatment with the PPARδ agonist GW501516 enhanced ANGPTL4 mRNA expression, resulting in an increased secretion of the C-terminal ANGPTL4 fragments to the cell culture supernatant. Intracellular expression of full-length ANGPTL4 was not affected [17]. Furthermore, we hypothesize that the enhanced cleavage of ANGPTL4 after α-13′-COOH treatment is responsible for the reduced amount of LPL monomers in cell culture supernatant. The availability of a sufficient amount of LPL monomers on the cell surface is essential for the formation of dimeric LPL structures and therefore the functionality of the enzyme [14]. It is generally accepted that ANGPTL4 inhibits LPL due to the conversion of catalytically-active LPL dimers to inactive monomers [17,32,34,38,54], although the exact mechanism is a matter of debate. Among others, the most prominent explanatory approach is that ANGPTL4 is initially activated by cleavage of its full-length form. Afterwards, the N-terminal fragment of ANGPTL4 catalyzes a conformational switch from LPL dimers to inactive monomers on the cellular surface [34,54]. However, Dijk et al. provided evidence for a different mechanism, showing that ANGPTL4 promotes the degradation of LPL monomers. Therefore, enzymatic activity of LPL would be reduced due to the lesser availability of monomeric structures for the formation of catalytically active dimers. In their experiments, CHO-pgsA-745 cells were co-transfected with LPL and ANGPTL4. Processing of ANGPTL4 – indicated by the detection of C-terminal ANGPTL4 fragments in supernatant – reduced the extracellular amount of LPL monomers due to enhanced LPL degradation inside the cell [37]. In a later investigation, cleavage of ANGPTL4 was identified as the essential part of this mechanism, as ANGPTL4 silencing remarkably diminished the degradation of LPL in primary adipocytes [55]. In our own experiments, stimulation of ANGPTL4 cleavage by α-13′-COOH also reduced the amount of LPL monomers in cell culture supernatant. However, the intracellular amount of monomeric LPL remained unchanged. This was also observed by Dijk et al. after co-plating of CHO-pgsA-745 cells transfected with either ANGPTL4 or LPL. In this experimental setting, enhanced ANGPTL4 cleavage only reduced the amount of LPL monomers in the culture medium, while intracellular LPL was not affected [37]. The authors concluded that ANGPTL4 cleavage might also promote extracellular degradation of LPL monomers, which could also represent an explanation for our results. This concept is supported by another investigation by Dijk et al., showing that ANGPTL4 promotes LPL degradation through a PCSK-dependent mechanism. Due to the occurrence of PCSK5 and PCSK6 on the cellular surface [56,57], degradation of LPL by PCSKs outside the cell also seems plausible [55]. Nevertheless, α-13′-COOH treatment could also promote the intracellular degradation of LPL, resulting in a diminished secretion of LPL monomers to the cell culture supernatant, although no change of the intracellular LPL level was detectable. However, there is no proof for this hypothesis. Based on the observations of Dijk et al. and our own results, we conclude that α-13′-COOH promotes the degradation of LPL monomers via induction of ANGPTL4 cleavage, although the exact location and mechanism remains unclear. Therefore, α-13′-COOH mediates its inhibitory effect on LPL protein expression via a post-translational ANGPTL4-dependent mechanism.

It was further shown that cleavage of ANGPTL4 also improves its inhibitory potential on LPL activity [37,54]. Co-transfection of CHO-pgsA-745 cells expressing full-length ANGPTL4 together with LPL strongly reduced LPL activity in cell lysates and cell culture medium compared with cells without ANGPTL4 expression. Western blots of the cell culture medium revealed that the highest reduction of LPL activity was accomplished in the samples in which ANGPTL4 cleavage products (C-terminal fragments) were also detectable. In parallel, the amount of LPL monomers was reduced [37]. This is perfectly in line with our own results. Here, α-13′-COOH treatment strongly reduced VLDL-induced LPL activity after short-term (1 h) and long-term (24 h) pre-incubation (Fig. 5). We therefore conclude that α-13′-COOH reduces the catalytic activity of LPL due to the diminished availability of LPL monomers in the culture medium, resulting from an induction of ANGPTL4 cleavage and subsequent LPL degradation. Short-term (1 h) and long-term (24 h) pre-treatment with α-TOH reduced LPL activity to nearly the same extent as α-13′-COOH (Fig. 5). However, the inhibitory effect of α-TOH was accomplished by a different mechanism and at much higher concentrations. As already described, α-TOH treatment reduced LPL mRNA expression, resulting in the subsequent reduction of LPL monomers in cell culture supernatant, at least after 24 h incubation. Previous studies have revealed that the transcriptional regulation of LPL expression is also linked to the regulation of its catalytic activity [52,53]. In an adipocyte model, treatment with the gastrointestinal hormone glucose-dependent insulinotropic polypeptide (GIP) together with insulin enhanced LPL mRNA and protein expression, resulting in a subsequent induction of LPL activity. These effects were diminished after treatment with the transcription inhibitor actinomycin D, showing that the transcriptional regulation of LPL expression was responsible for the observed effects on LPL activity [53]. In line with this concept, a further investigation in adipocytes isolated from dexamethasone-treated Sprague-Dawley rats showed a reduction of LPL mRNA expression followed by a subsequent reduction of LPL activity [52]. We therefore conclude that α-TOH reduces the catalytic activity of LPL due to its inhibitory effect on LPL mRNA expression.

Changes in LPL activity are closely connected to changes in intracellular lipid accumulation, since LPL represents a key enzyme for the cellular supply with free-fatty acids from triglyceride-rich lipoproteins. Elevations of plasma VLDL in patients with diabetes mellitus type 2 or metabolic syndrome are associated with an increased risk of atherosclerosis [58]. It has also been shown that VLDL enhances lipid accumulation in vitro, making it a good test stimulus for our THP-1 macrophage model [59]. Based on the results of our previous experiments, we hypothesized that α-TOH and α-13′-COOH can prevent excessive intracellular lipid accumulation in human THP-1 macrophages in the presence of VLDL oversupply. Our hypothesis is strengthened by the observations of Wallert et al. on oxLDL-induced neutral lipid accumulation in THP-1 macrophages. Here, oxLDL oversupply increased cellular neutral lipid accumulation, while co-incubation with 5 μM α-13′-COOH dampened the effect [8]. Unfortunately, no data on the influence of α-TOH on neutral lipid accumulation in the presence of a comparable lipoprotein stimulus was available. However, Schmölz et al. showed that α-TOH as well as α-13′-COOH treatment alone enhanced neutral lipid accumulation in THP-1 macrophages [12]. As expected from our previous results, α-13′-COOH treatment diminished VLDL-induced lipid accumulation after 24 h pre-incubation, although there was no effect after 1 h pre-incubation (Fig. 6). We therefore conclude that α-13′-COOH reduced VLDL-induced lipid accumulation by an

ANGPTL4-dependent inhibition of LPL activity. Furthermore, a sufficiently long pre-incubation time with α-13′-COOH seems important for the development of its regulatory potential on lipid accumulation. However, α-TOH revealed contrary results to what would have been expected from our previous investigations. Although the catalytic activity of LPL was reduced, no reduction of VLDL-induced lipid accumulation was observed after 1 h and 24 h pre-incubation with α-TOH (Fig. 6). However, previous studies have shown that LPL is able to enhance cellular lipoprotein uptake via pathways independent of its catalytic activity, requiring LPL protein as a molecular bridge between lipoproteins and cellular receptors or proteoglycans [60–64]. As an example, Beisiegel et al. showed that HepG2 cells and fibroblasts absorbed triglyceride-rich lipoproteins – including chylomicron remnants and VLDL – via a low-density lipoprotein receptor-related protein (LRP)-dependent mechanism. In this study, LPL enhanced the binding of both lipoproteins to LRP. This bridging effect appeared to be independent of the catalytic activity of LPL but to be dependent on the protein itself [60]. In order to investigate the LPL bridging effect in vivo, Merkel et al. created a catalytically inactive form of human LPL by introducing a mutation in its catalytic domain. Selective expression of this inactive LPL in the muscle of *LPL* $^{-/-}$ mice resulted in a reduction of plasma VLDL and triglyceride levels by 33% compared with control mice. In line with this, triglyceride concentration in the muscle of mice expressing catalytically inactive LPL was also increased [61]. Based on the observations of Beisiegel et al. and Merkel et al., we conclude that the presence or absence of LPL protein on the cellular surface seems to be another important factor to modulate neutral lipid accumulation inside the cell, independent of its catalytic activity. Although α-TOH and α-13′-COOH inhibited LPL activity, only α-13′-COOH also diminished intracellular lipid accumulation, at least after long-term pre-incubation. A plausible explanation for this difference may be found in the comparison of LPL protein in the cell culture supernatant: while the amount of LPL monomers was still reduced after 48 h α-13′-COOH treatment, the secreted amount of LPL was restored to the control level in the α-TOH-treated samples. Based on the aforementioned studies, LPL-VLDL bridging potentially may increase intracellular lipid accumulation in the α-TOH-treated samples, although the catalytic activity of LPL was inhibited. However, it cannot be excluded that α-TOH enhances intracellular lipid accumulation through an LPL-independent mechanism.

Due to the promising effects of α-13′-COOH in preventing excessive lipid accumulation in human THP-1 macrophages, we were further interested in ascertaining the signaling mechanism underlying these effects. It is well established that transcriptional regulation of ANGPTL4 is predominantly mediated by PPARs [17,47–49]. The peroxisome proliferator-activated receptor δ represents the predominant PPAR form in human THP-1 macrophages, because its expression is increased during monocyte differentiation compared with the other PPAR forms [50]. Stimulation of THP-1 macrophages with PPARδ agonists also increased ANGPTL4 mRNA expression [17]. Interestingly, several studies have demonstrated that VLDL-induced lipid accumulation in macrophages is attenuated by a pre-treatment with PPARδ agonists [59,65]. However, the observations on this topic are contradictory. We hypothesize that α-13′-COOH can affect the LPL system as well as intracellular lipid accumulation through a PPARδ-dependent mechanism. In order to test this hypothesis, the effect of co-incubation of α-13′-COOH with GSK3787 – a selective antagonist of PPARδ [51] – was investigated on mRNA expression and at the functional level, i.e. measurement of LPL activity and neutral lipid accumulation. Although co-incubation with GSK3787 attenuated the induction of ANGPTL4 mRNA expression, GSK3787 failed to reduce the inhibitory effects of α-13′-COOH on LPL activity and neutral lipid accumulation. We therefore concluded that the induction of ANGPTL4 mRNA expression by PPARδ is at least one – but not the crucial – mechanism for α-13′-COOH-dependent regulation of LPL-mediated lipid homeostasis. However, it cannot be excluded that the increase of ANGPTL4 mRNA expression in the combination of α-13′-COOH and GSK3787 is still sufficient to induce the inhibitory effects of α-13′-COOH at the functional level. Based on the sparse knowledge about the interaction of α-13′-COOH with intracellular signaling pathways, we can only speculate about an alternative route to the PPARδ-mediated mechanism. In a previous investigation, Schmölz et al. provided convincing evidence that the specific effect of α-13′-COOH depends on its complete molecular structure, including the chromanol ring system, aliphatic side chain and the carboxylic group. If one of the three components was missing, no regulatory effect of α-13′-COOH could be observed [31]. Therefore, Schmölz et al. proposed the hypothesis of the existence of a specific, hitherto-unidentified cellular receptor for α-13′-COOH and other LCMs of vitamin E [12,31]. If this concept holds true, α-13′-COOH would be able to activate indirect signal transduction pathways, among others by G protein-coupled receptors or the activation of ion channels. Especially the activation of ion channels would be of interest, since calcium represents an important co-factor for the enzymatic activity of PCSKs [66,67]. In addition, it has been shown that ANGPTL4 promotes the degradation of LPL through a PCSK-dependent mechanism, independent of its transcriptional regulation [55]. Overall, the direct regulation via PPARδ seems unlikely, but based on the combination of the mentioned observations, α-13′-COOH could probably affect LPL-mediated lipid homeostasis via other signaling pathways, such as calcium-dependent mechanisms. However, further studies are needed to prove this hypothesis.

Despite having shown convincing effects of α-13′-COOH on the ANGPTL4-dependent regulation of cellular lipid homeostasis, there is still a limitation of the overall story. Although there are a variety of hints that ANGPTL4 is responsible for the α-13′-COOH-mediated effects on cellular lipid homeostasis, final validation via small interfering RNA (siRNA) knockdown of *ANGPTL4* is missing. Unfortunately, we were not able to establish a reliable *ANGPTL4* knockdown model in human THP-1 macrophages that can be combined with our LPL activity assay as well as the measurement of neutral lipid accumulation. The knockdown of *ANGPTL4* was impeded by the effect of serum depletion on ANGPTL4 mRNA expression. Hence, there was no detectable difference between basal expressions of *ANGPTL4* in the vehicle control compared with the *ANGPTL4* siRNA-transfected sample under serum-free conditions. In our opinion, ANGPTL4 mRNA expression level was already too low due to serum depletion. Furthermore, initial experiments (data not shown) revealed that *ANGPTL4* siRNA knockdown was unable to compensate the induction of ANGPTL4 mRNA expression by α-13′-COOH. Further, we like to point out that the human THP-1-like macrophage model used for the experiments presented here cannot cover the complex biochemical, physiological or metabolic processes affecting LCM functionality in living organisms. We are also aware that as an acute myeloid leukemia cell line, THP-1-like macrophages may have several differences compared to primary human macrophages, such as peripheral blood mononuclear cells (PBMCs). Nevertheless, since the main aim of the present study was to generate first data on fundamental regulatory processes mediated by the α-TOH derived LCM α-13′-COOH, the use of human THP-1-like macrophages as a reliable and easy to handle in vitro model was justified. Hence, the results generated in this study allow, at least in parts, conclusions to the in vivo situation.

5. Conclusion

Here we provide new insights into the regulatory role of α-13′-COOH and its vitamin precursor α-TOH in another facet of lipid metabolism. Interestingly, the two compounds affected the LPL system through different regulatory mechanisms: While α-TOH affected LPL expression via transcriptional regulation, α-13′-COOH triggered post-translational regulation of LPL via ANGPTL4. Furthermore, both molecules effectively reduced the catalytic activity of LPL. However, only α-13′-COOH was able to protect human THP-1 macrophages against excessive lipid accumulation in the presence of VLDL oversupply (Fig. 8).

As already observed by others [8,12,31], much higher doses of α-TOH (100 μM) were required to preserve regulatory effects compared

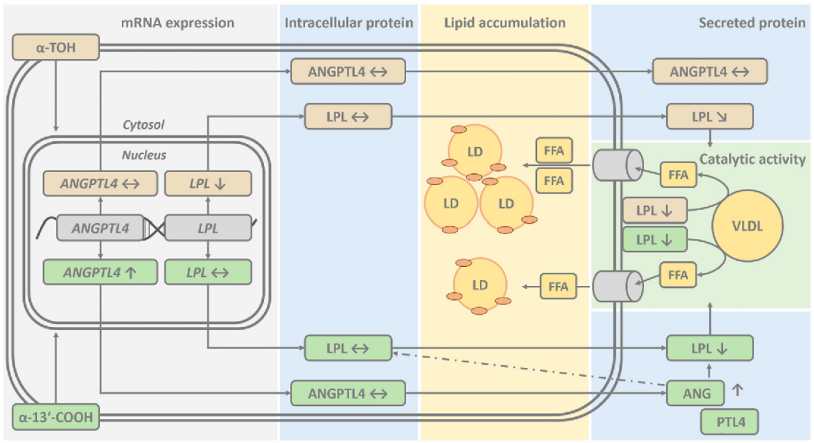

Fig. 8. Schematic overview on the proposed regulatory activities of α-13′-COOH and its precursor α-TOH on LPL-dependent foam cell formation of macrophages. We found that α-13′-COOH (5 μM) increases the expression of angiopoietin-like 4 (ANGPTL4) mRNA in human THP-1 macrophages in a time- and dose-dependent manner, while α-TOH (100 μM) shows no effect. In contrast, the mRNA of lipoprotein lipase (LPL) is not influenced by α-13′-COOH, but α-TOH reduces expression of LPL mRNA. Both compounds also reveal different effects on protein levels: while α-13′-COOH reduces the amount of secreted LPL protein via cleavage-mediated activation of ANGPTL4 protein, the amount of secreted LPL in the α-TOH-treated samples was diminished via direct transcriptional regulation. Further, both compounds reduce the catalytic activity of LPL. However, only α-13′-COOH (5 μM) but not α-TOH (100 μM) attenuate VLDL-induced lipid accumulation in the presence of VLDL. Abbreviations: FFA, free fatty acid; LD, lipid droplet; VLDL, very-low density lipoprotein.

with α-13′-COOH (5 μM). Although their exact mode of action remains unknown, our results support the concept that the LCMs of vitamin E – including α-13′-COOH – represent the activated and therefore functional forms of their vitamin precursors. Since only α-13′-COOH and not α-TOH prevented excessive lipid accumulation in macrophages, we also provide evidence for an atheroprotective potential of this regulatory metabolite due to the reduction of VLDL-induced foam cell formation. Nevertheless, further studies are required to elucidate the underlying regulatory pathways and the physiological relevance of α-13′-COOH.

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CRediT authorship contribution statement

Stefan Kluge: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Martin Schubert:** Writing – review & editing. **Lisa Börmel:** Investigation. **Stefan Lorkowski:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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7.2 Manuscript II

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Method Article

Simple and rapid real-time monitoring of LPL activity *in vitro*

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A B S T R A C T

Since elevated plasma triglycerides are an independent risk factor for cardiovascular diseases, lipoprotein lipase (LPL) is an interesting target for drug development. However, investigation of LPL remains challenging, as most of the commercially available assays are limited to the determination of LPL activity. Thus, we focused on the evaluation of a simple *in vitro* real-time fluorescence assay for the measurement of LPL activity that can be combined with additional cell or molecular biological assays in the same cell sample. Our procedure allows for a more comprehensive characterization of potential regulatory compounds targeting the LPL system.

The presented assay procedure provides several advantages over currently available commercial *in vitro* LPL activity assays:

1. 12-well cell culture plate design for the simultaneous investigation of up to three different compounds of interest (including all assay controls).
2. 24 h real-time acquisition of LPL activity for the identification of the optimal time point for further measurements.
3. Measurement of LPL activity can be supplemented by additional cell or molecular biological assays in the same cell sample.

A R T I C L E I N F O
Method name: Cell culture based real-time fluorescence assay for the measurement of LPL activity
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Abbreviations: ANGPTL, angiopoietin-like; FBS, fetal bovine serum; FFA, free fatty acid; FI, fluorescence intensity; LPL, lipoprotein lipase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PPAR, proliferator-activated receptor; PSG, L-glutamine-penicillin-streptomycin; RFU, relative fluorescence units; VLDL, very low-density lipoprotein.

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Specification Table

Subject area:	*Biochemistry, Genetics and Molecular Biology*
More specific subject area:	*Investigation of lipoprotein lipase activity*
Method name:	*Cell culture based real-time fluorescence assay for the measurement of LPL activity*
Name and reference of original method:	*Name: Abcam Lipoprotein Lipase Assay Kit (Fluorometric) (ab204721)* *Source:* [1]

Method details

Background

Lipoprotein lipase (LPL) mediates the release of free fatty acids (FFAs) from triglyceride-rich lipoproteins, like chylomicrons and very low-density lipoproteins (VLDL). Therefore, LPL represents a key enzyme for the regulation of cellular lipid homeostasis by providing FFAs for cellular energy supply and intracellular energy storage as well as a control mechanism for plasma triglyceride levels [2]. The activity of LPL is primarily regulated by post-translational modifications. The family of angiopoietin-like (ANGPTL) proteins, whose expression is controlled by peroxisome proliferator-activated receptors (PPARs), appeared as potent physiological inhibitors of LPL activity [3–5]. LPL dysfunction or dysregulation can result in elevated plasma triglycerides [6,7]. There is growing evidence that elevated plasma triglycerides are an independent risk factor for cardiovascular diseases [8,9], making the LPL system an interesting target for drug development [10,11]. Hence, determination of LPL activity is a useful tool for the identification of potential lead compounds from natural or synthetic origins. Commercially available kits for the measurement of LPL activity are based on radiolabeled (^{3}H or ^{14}C), fluorogenic or chromogenic substrates [12–14]. These substrates are degraded by LPL and their reaction products can be detected at defined times [15]. Unfortunately, most commercial kits are optimized for post heparin plasma samples and are therefore not suitable for initial characterization of potential drug compounds in *in vitro* systems. Next, the few commercial LPL assays that are optimized for *in vitro* application require cell harvesting and homogenization. Thus, cells cannot be used for further cell or molecular biological investigations, which would allow a more comprehensive characterization of the respective test compound [1,16]. For example, the measurement of LPL activity combined with subsequent RT-qPCR or Western blot analyses can serve as a useful tool for the identification of transcriptional regulators of LPL activity.

For this reason, we decided to develop a simple cell culture based real-time fluorescence assay for the measurement of LPL activity that can be combined with cell and molecular biological analyses of the same cell sample. In our method, LPL activity is measured using a fluorescently labeled and quenched LPL substrate in combination with isolated VLDL for stimulation of LPL activity.

Required reagents and equipment

(1) *VLDL isolation*
- 50 ml fasted blood sample
- EDTA monovettes
- Centrifuge applicable for at least 1870 × *g*
- Ultracentrifuge thick wall tubes
- Ultracentrifuge
- Ultracentrifuge rotor applicable for at least 200,000 × *g*

(2) *Cell culture*
- 12-well cell culture plates
- Adherent cells
- Fetal bovine serum (FBS)
- L-glutamine-penicillin-streptomycin (PSG)
- Cell type specific cell culture medium
- Cell type specific phenol red free cell culture medium (for THP-1 macrophages: RPMI-1640, R7509, Sigma Aldrich)

- VLDL isolated form blood samples of healthy volunteers (alternatively: order commercially available VLDL)
- Fluorescently labeled, quenched LPL substrate (ab214552, Abcam, Cambridge UK)
- Orlistat (O4139, Sigma Aldrich; negative control)

(3) *Fluorescence-based real-time measurement of LPL activity*

- Microplate reader coupled to an atmospheric control unit applicable for fluorescence measurements (Ex/Em = 485/520 nm)
- Microplate reader temperature: 37 °C
- CO2 concentration: 5% (v/v)

Procedure

The description of the following experimental procedure will be illustrated by the human THP-1 macrophage cell model. However, this method may be suitable for various adherent cell lines that express active LPL.

(1) *Isolation of VLDL (alternatively: order commercially available VLDL)*

Note: VLDL is an essential part of the assay procedure. It is used as positive control, because stimulation of LPL activity by VLDL has been already established as positive control for plasma measurements of LPL activity [12].

- Use 50 ml blood obtained from fasted donors with plasma triglyceride concentrations of > 0.90 mmol/l

Note: Plasma triglyceride concentrations of > 0.90 mmol/l was previously described as a suitable range for VLDL-based LPL assays [12].

- Collect the blood in 9 ml EDTA-monovettes (02.1066.001, Sarstedt)
- Centrifuge blood samples at 1870 × g for 10 min at 15 °C for plasma separation
- Transfer plasma into 4 ml thick-wall polycarbonate tubes (355,645, Beckmann Coulter) for ultracentrifugation
- Ultracentrifugation is performed for 4 h at 15 °C and 269 200 × g (50,000 rpm, used rotor: Type 50.4 Ti, Beckman Coulter)
- Collect separated VLDL in 2 ml tubes

Note: The separated VLDL phase is very narrow and can easily be mixed with the plasma fraction below. Be careful not to shake the thick-wall tubes when taking them out of the ultracentrifuge. Use a 1 ml pipette for collecting the VLDL phase by placing the pipette tip on the tube wall and moving it carefully around the tube.

- Determine the protein concentration of the VLDL samples (*e.g.*, Lowry or Bradford assay)
- Isolated VLDL can be stored under nitrogen atmosphere at 4 °C

Note: It is necessary to utilize the isolated VLDL within one week for the respective experimental procedures to ensure high VLDL quality and to avoid lipid oxidation.

(2) *Cell culture*

- The assay procedure described below is based on the use of 12-well cell culture plates (92,012, TPP Techno Plastic Products, Trasadingen, Switzerland)
- Use 1 × 106 THP-1 monocytes per well for macrophage differentiation (add 100 ng/ml phorbol-12-myristate-13-acetate (P1585, Sigma Aldrich) and 50 μmol/l β-mercaptoethanol (4227.3, Carl Roth) together with RPMI-1640 (R8758, Sigma Aldrich) cell culture medium supplemented with 10% (v/v) FBS Superior (S0615, Sigma Aldrich) and 0.1% (v/v) PSG solution (G1146, Sigma Aldrich))

Note: The number of wells used for the assay depends on the experimental design. However, there are four fixed controls included in each assay run to ensure reliability of the procedure: (i) an untreated control, (ii) the orlistat negative control without VLDL, (iii) the VLDL positive control, and (iv) the orlistat negative control in combination with VLDL. Keep in mind to include these four controls in your experimental design. The current 12-well assay design including all assay controls allows the simultaneous analysis of up to three different test compounds.

- THP-1 monocytes are differentiated for 96 h in 2 ml cell culture medium per well
- Remove cell culture supernatant from the fully matured THP-1 macrophages
- Wash cells twice with phosphate-buffered saline (PBS)
- Pre-incubate cells according to the respective experimental procedure (here for 24 h) in 1 ml phenol red-free RPMI-1640 medium (R7509, Sigma Aldrich) under serum-free conditions
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: It is necessary to replace standard RPMI-1640 cell culture medium by phenol red-free RPMI-1640 for incubation to avoid fluorescence interferences. For the basic assay procedure without any test compounds, cells are only pre-incubated with 50 µM orlistat in the respective wells for 24 h.

- Add VLDL in a concentration equivalent to 50 µg/ml protein together with 0.5 µl quenched, fluorescently labeled LPL substrate to the pre-incubated cells
- Shake the cell culture plate carefully to ensure homogenous distribution of the compounds

Note: The LPL substrate used for the experimental procedure is a standardized, commercially available product from Abcam (Cambridge, UK ab214552). It is a component of the commercial LPL assay kit offered by the company and therefore validated for reliable functionality. Nevertheless, we recommend to pooling LPL substrates of different batches to reduce variability between measurements. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured fluorescence intensity (FI) values are proportional to the amount of hydrolyzed substrate and thus LPL activity. The use of commercially available substrates for determination of LPL activity has also been described in other methodical approaches [12,13].

(3) *Fluorescence based real-time measurement of LPL activity*

Note: In our experimental setup, the FLUOstar Omega microplate reader coupled to an atmospheric control unit (BMG Labtech, Ortenberg, Germany) was used for FI determination. As an initial step, a suitable measurement procedure should be prepared for the respective experimental design of every assay procedure. In our approach, we determined FI values of the used wells hourly over 24 h at Ex/Em = 485/520 nm (recommended wavelength for the LPL substrate). As an additional preparation step, a temperature of 37 °C and a CO_2 concentration of 5% (v/v) should be set at least one hour before each measurement.

- Place the cell culture plate in the plate reader and start the prepared measurement procedure.
- The determined FI values can be used for the assessment of LPL activity for any test compound.
- Adherent cells can be used for subsequent investigations.

Note: The described assay conditions do not require harvesting of the cells. Thus, LPL activity measurement can be supplemented by additional cell or molecular biological analyses for a more comprehensive characterization of the test compounds. We have currently performed Nile red staining and cell viability assays after the determination of LPL activity. However, the combination with other molecular biological methods, such as Western blot, RT-qPCR or any other *in vitro* application is possible in principle.

Method validation

The aim of this study was to establish a simple fluorescence-based *in vitro* assay to allow the initial characterization of potential regulatory compounds targeting the LPL system. We decided to develop this procedure because commercially available LPL assays were not suitable for this demand.

Preliminary experiments revealed that the standard RPMI-1640 cell culture medium had to be replaced by phenol red-free RPMI-1640 for cell incubation to avoid fluorescence interferences. To enable determination of LPL activity, we used a quenched, fluorescently labeled LPL substrate (similar approach as described in [12,13]) in our incubation procedure. The quenched substrate fluoresces upon hydrolysis by LPL, so that the measured FI values are proportional to the amount of hydrolyzed substrate and thus LPL activity. Further, we decided to use VLDL as positive control, because stimulation of LPL activity by VLDL has already been shown in plasma measurements [12]. In addition, orlistat, a well-established and clinically used LPL inhibitor [17–19] was added as a negative control.

For the initial establishment of the assay procedure, human THP-1 macrophages were treated as described in the section "Supplemental Material/and or additional information". After 24 h real-time measurement, we noticed that incubation with VLDL (in a concentration equivalent to 50 µg/ml protein) enhanced the measured FI values, indicating increased LPL activity, in a time dependent manner. Fluorescence intensity values of the untreated control and the VLDL-treated sample (positive control) evolved in a significantly different range ($p < 0.01$) (Fig. 1(A)). Maximum FI values have been determined after 24 h as an increase to 268.75 ± 24 relative fluorescence units (RFU) in the VLDL-incubated sample compared to 187 ± 14.5 RFU in the untreated control. As expected, orlistat (negative control) significantly blocked LPL activity compared to VLDL-treatment and the untreated control. Fluorescence intensity values of the VLDL-incubated sample and the combination of VLDL and orlistat evolved over time in a significantly different range ($p < 0.001$). After 24 h, FI value for the cells incubated with a combination of VLDL and orlistat was determined at 150 ± 1.3 RFU and as significantly lower ($p < 0.01$) compared to the VLDL-incubated cells (268.75 ± 24 RFU) (Fig. 1(A)). These results indicate that our methodical approach and the used assay controls are suitable for the *in vitro* measurement of LPL activity.

As a further validation of the established assay procedure, we decided to use GW0742 as a reference test compound. GW0742 is a well-studied PPAR-δ agonist [20,21], that enhances the expression of ANGPTL4 mRNA [22], a potent physiological and endogenously produced inhibitor of LPL. Consequently, stimulation of THP-1 macrophages with the PPAR-δ agonist GW0742 should enhance ANGPTL4 expression, in turn causing a reduction of cellular LPL activity [5]. To confirm the reported effects of GW0742 under our conditions, human THP-1 macrophages were treated with 100 nM GW0742 and harvested at different time points for RT-qPCR analysis. As expected, GW0742 enhanced ANGPTL4 mRNA expression already after 1 h by approximately 20-fold ($p < 0.001$) and after 24 h by approximately 200-fold ($p < 0.001$) (Fig. 1(B)). To examine the effects of GW0742 on cellular LPL activity, we expanded our initial working procedure by an additional 24 h pre-incubation with 100 nM GW0742. In line with our initial experiment, we were able to generate similar FI values for untreated control cells, VLDL-incubated cells as well as cells cultured with orlistat alone or in combination with VLDL. GW0742 treatment slightly, but not significantly increased basal LPL activity (206 ± 20 RFU *vs.* 230 ± 16 RFU) compared to the untreated control. However, combination of GW0742 and VLDL did significantly reduce the induction of LPL activity by VLDL ($p < 0.05$). The strongest reduction was achieved after 24 h, where GW0742 reduced FI values of VLDL incubated cells from 291.3 ± 29 RFU to the control level of 196.6 ± 20 RFU (Fig. 1(C)). In summary, the observed effect of GW0742 on cellular LPL activity is consistent to the literature.

For the combination of the LPL assay procedure outlined here with additional cell or molecular biological methods, we studied cell viability at the end of the LPL activity measurement. For this, methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed after completing the 24 h real-time measurement. None of the applied compounds did significantly reduce cell viability compared to the untreated control. Treatment with orlistat led to a slightly but not significantly reduced cell viability of 85% of the control, representing the lowest viability in our experimental setup (Fig. 1(D)). According to ISO 10993-5:2009, a reduction of cell viability by 15% is not regarded as a cytotoxic effect [23]. For final validation of the assay procedure, we combined our LPL assay

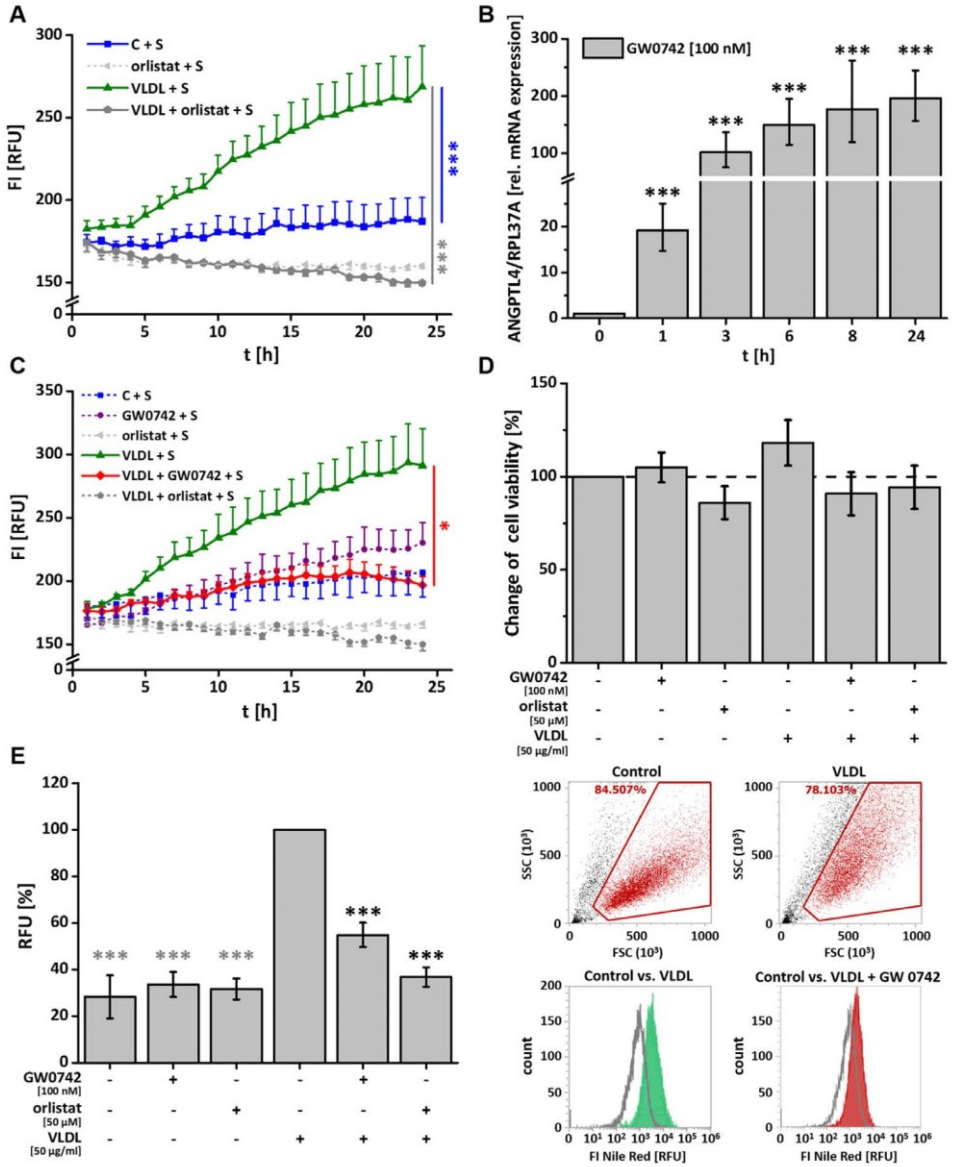

Fig. 1. *Establishment and validation of an in vitro real-time fluorescence assay for the measurement of LPL activity.* (A) Initial establishment of the LPL assay procedure. Human THP-1 macrophages were pre-incubated with 50 µM orlistat (negative control). After 24 h, VLDL (positive control, protein concentration of 50 µg/ml) and the fluorescently labelled LPL subtrate were added to the corresponding wells. Fluorescence intensity (FI) of each well was determined hourly over 24 h at Ex/Em = 485/520 nm ($n = 4$; *** $p < 0.001$ *vs.* VLDL incubation). All further experiments were performed for assay validation, using GW0742 as reference test compound. (B) RT-qPCR of human THP-1 macrophages to investigate ANGPTL4 (LPL inhibitor) mRNA expression after GW0742 treatment ($n = 4$, *** $p < 0.001$, *vs.* untreated control). ANGPTL4 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions (data not shown). (C) LPL activity assay with GW0742 as test compound ($n = 3$, * $p < 0.05$ *vs.* VLDL incubation). (D) MTT assay for the assessment of cell viability after measurement of LPL activity ($n = 3$). (E) Measurement of the accumulation of neutral lipids accumulation by flow cytometry using Nile red staining after completion of the real-time LPL activity assay ($n = 3$, *** $p < 0.001$, *vs.* VLDL treatment).

with a subsequent measurement of neutral lipid accumulation by flow cytometry using Nile red staining. This experimental setting was chosen from reports on VLDL-induced cellular accumulation of neutral lipids via LPL [24,25]. Further, ANGPTL4 has been shown to reduce the uptake of triglyceride-derived fatty acids from VLDL by human THP-1 macrophages [26]. Consequently, VLDL treatment should enhance neutral lipid accumulation, while GW0742 treatment should prevent this. As expected, incubation of THP-1 macrophages with VLDL induced accumulation of neutral lipids by almost threefold compared to the untreated control. Co-incubation with GW0742 significantly reduced VLDL-induced accumulation of neutral lipids to 54% ($p < 0.001$). Co-incubation with orlistat resulted also in a significant reduction of VLDL-induced accumulation of neutral lipids to 36% ($p < 0.001$). As expected, the relative amount of neutral lipids in cells treated with GW0742 or orlistat but without VLDL did not differ from the control (Fig. 1(E)). The obtained results confirm that our LPL assay can be easily combined with other cell and molecular biological methods to produce more comprehensive information on the interaction of a compound of interest with the cellular LPL system.

Conclusion

We here provide a simple and rapid fluorescence-based *in vitro* assay for the assessment of the interactions of test compounds with the LPL system. The assay procedure provides several advantages over currently available *in vitro* LPL assays: (i) 12-well cell culture plate design for the simultaneous investigation of up to three different test compounds (including all assay controls); (ii) 24 h real-time acquisition of LPL activity data for the identification of the optimal time point for further measurements; and (iii) LPL activity measurement can be complemented by additional cell and molecular biological analyses using the same cell samples. Nevertheless, we are aware that the current assay design has limitations and needs further improvements:

(i) In our experiments, VLDL was isolated from only a single normolipidemic male donor with plasma triglyceride concentrations of 0.90 mmol/l. However, the approach could be improved by using a mixture of VLDL obtained from multiple donors as described in [12]. Here, samples from ten normolipidemic donors (plasma triglycerides < 1.75 mmol/l) were pooled to create a more representative mean VLDL substrate for their assay procedure. Further, the use of a VLDL pool from different donors may also reduce the variation of the lipid and apolipoprotein composition between individuals.

(ii) For further optimization of VLDL composition, the triglyceride content of the VLDL should be determined before. Di Filippo and coworkers used seven VLDL pools with varying triglyceride concentrations ranging from 0.45 to 3.45 mmol/l for the determination of LPL activity in post-heparin plasma [12]. The authors report that LPL activity reached a steady level in the range between 1.5 and 2.2 mmol/l, while lower concentrations (< 0.90 mmol/l) decreased LPL activity and high concentrations (> 2.7 mmol/l) slightly increased LPL activity in post-heparin plasma. To avoid distortion of the measured LPL activity by suboptimal triglyceride concentrations of the applied VLDL, the authors determined 1.8 mmol/l as the optimal triglyceride concentration for their assay. Hence, optimization of the triglyceride concentrations in the VLDL could improve our assay.

(iii) The assessment of LPL activity in the current assay design is based on the comparison of FI values for the different compounds and controls at various times. This might be sufficient for a first impression of the effect of compounds on LPL activity and is therefore suitable for the intention of our assay procedure. Nevertheless, exact quantification of LPL activity based on the calculation of the released amount of substrate over time, as it has already been described for plasma measurements [1,12,13], should be an aim for future improvements. Unfortunately, no detailed information about their fluorescently labeled LPL substrate (concentration, composition, exact chemical name/structure etc.) is available from the supplier (Abcam, Cambridge UK). It was therefore not possible to adapt the procedure for the use of standard calibration curves as described in the assay manual to our conditions [1].

(iv) For the here presented LPL activity assay, a mean coefficient of variation (CV) for inter-assay variability was 10.6% for low (untreated control) and 12% for high values (VLDL treatment).

Although both CVs are below 15%, which is in general acceptable for biological assays [27], inter-assay variability is an issue, in particular for high FI values. In general, the higher the measured FI values the higher the variation between each measurement. We were able to partially reduce this problem using a pool of different batches of the LPL substrate. Given the fact that the current assay procedure is only designed for initial compound screening and not as a diagnostic tool, the variation between each measurement seems acceptable. Nevertheless, reproducibility and the accuracy of the assay procedure should further be improved.

(v) All experiments and optimizations for the current assay procedure were performed with human THP-1 macrophages. However, to fully understand the global impact of a given drug on lipolysis the use of further LPL-expressing cells, like adipocytes, may be necessary.

Despite of its limitations, our assay design can serve as a reliable tool for *in vitro* measurements of the effects of test compounds modulating the activity of the LPL system.

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Declaration of Competing Interest

The authors declare to have no competing interests.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.100865.

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7.3 Manuscript III

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Long-chain metabolites of vitamin E: Interference with lipotoxicity via lipid droplet associated protein PLIN2

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ABSTRACT

The long-chain metabolites of vitamin E (LCM) emerge as a new class of regulatory metabolites and have been considered as the active compounds formed during vitamin E metabolism. The bioactivity of the LCM is comparable to the already established role of other fat-soluble vitamins. The biological modes of action of the LCM are far from being unraveled, but first insights pointed to distinct effects and suggested a specific receptor, which in turn lead to the aforementioned hypothesis. Here, a new facet on the interaction of LCM with foam cell formation of THP-1 macrophages is presented. We found reduced levels of mRNA and protein expression of lipid droplet associated protein PLIN2 by α-tocopherol (α-TOH), whereas the LCM and the saturated fatty acid, stearic acid, increased expression levels of PLIN2. In a lipotoxic setup (0–800 μM stearic acid and 0–100 μM α-TOH or 0–5 μM α-13′-COOH) differences in cellular viability were found. A reduced viability was observed for cells under co-treatment of α-TOH and stearic acid, whereas an increased viability for stearic acid incubation in combination with α-13′-COOH was observed. The striking similarity of PLIN2 expression levels and worsened or mitigated lipotoxicity, respectively, revealed a protective effect of PLIN2 on basal stearic acid-induced lipotoxic conditions in PLIN2 knockdown experiments. Based on our results, we conclude that α-13′-COOH protects cells from lipotoxicity, at least partially via PLIN2 regulation.

Herewith another facet of LCM functionality was presented and their reputation as regulatory metabolites was further established.

1. Introduction

Recently, a new perspective on vitamin E and its metabolism was postulated [1]. The metabolites, which are formed during hepatic degradation of tocopherols and tocotrienols, were thought to be just products of vitamin E excess. However, currently a change of this paradigm suggests that in particular the long-chain metabolites (LCM), the first metabolites occurring in vitamin E metabolism, may be the activated and thereby functional molecules in the family of vitamin E derivatives. This concept is appealing, as it has already been accepted for other fat soluble vitamins, such as vitamin A or D [2–4].

Vitamin E is a collective term summarizing eight highly similar structures, all of which consist of a chromanol ring-system and an aliphatic side-chain. However, the different forms of vitamin E differ in the saturation of their side-chain, leading to the saturated tocopherols (TOH) and the unsaturated tocotrienols (T3). The methylation pattern of the chromanol ring-system determines the α-, β-, γ- or δ-forms. The hepatic metabolism of vitamin E is principally independent of these features, whereas its efficiency highly depends on the type of methylation (α-TOH is the form with the lowest catabolic rate [5]). In more detail, an oxidative modification of the side-chain via cytochrome P450-dependent enzymes (CYP4F2/CYP3A4) leads to the formation of the LCM, which are in the case of α-TOH, α-13′-hydroxychromanol (α-13′-OH) and α-13′-carboxychromanol (α-13′-COOH). A more detailed overview is provided in a recent review [5].

The biological action of the LCM is far from being unraveled.

Abbreviations: α-13′-OH, α-13′-hydroxychromanol; α-13′-COOH, α-13′-carboxychromanol; LCM, long-chain metabolites of vitamin E; TOH, tocopherol; T3, tocotrienols

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However, several comprehensive studies have been published in recent years, mainly covering the topics of interaction of the LCM with inflammation [6–10], cancer [11,12], handling of pharmaceuticals [13], and macrophage foam cell formation [14], which is a hallmark in the progression of atherosclerosis. In brief, macrophages within the arterial wall are loaded with lipids, mainly originating from oxidized lipoprotein particles (e.g., low density lipoproteins (LDL); for more details, the reader is referred to [15]). These lipids can be stored in so-called cytosolic lipid droplets. Through the light microscope, a cell filled with lipid droplets appears to be foamy, which was eponymous for foam cells. The lipid droplets are organelles composed of a phospholipid monolayer, a lipid core containing triglycerides and sterol esters, as well as proteins, which are integrated in the phospholipid monolayer [16]. One of these proteins is PLIN2 (formerly adipophilin or adipose differentiation related protein (ADRP)), which was first identified by Jiang et al. in 1992 [17,18].

When the lipid loading capacity of cells is exceeded, a mechanism called lipotoxicity is induced [19]. The impaired cellular signaling, as well as mitochondrial and ER dysfunction may lead to cell death [20]. In the case of atherogenesis, this causes the formation of the necrotic lipid core of atherosclerotic plaques. In the initial phase of lipotoxicity, the storage of lipids in lipid droplets is protective as free fatty acids are esterified to triglycerides and are thus removed from active signaling [19,21].

To get a deeper insight into the biological actions of the LCM, we focused on the regulatory effects of α-13′-COOH on the expression of PLIN2, its interference with stearic acid-induced lipotoxicity and the possible connections between both mechanisms.

2. Materials and methods

2.1. Chemicals

If not indicated otherwise, chemicals were purchased from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), Fisher Scientific (Schwerte, Germany), or Merck Millipore (Darmstadt, Germany).

2.2. Cell culture

THP-1 monocytes (ATCC, Manassas, VA), cultivated in RPMI 1640 supplemented with 10% (v/v) FBS and 0.1 mg/ml penicillin/streptomycin/L-glutamine [22] were differentiated into macrophages using 100 ng/ml phorbol-12-myristate-13-acetate (PMA) and 50 μM β-mercaptoethanol [23]. After 96 h, macrophages were incubated with serum-free supplemented medium and the test compounds as indicated in the figures and were harvested for further processing as described below.

2.3. Incubation

Stearic acid (C18:0; Alfa Aesar, Haverhill, MA) was dissolved in pure ethanol and complexed to fatty acid-free bovine serum albumin (Sigma-Aldrich) at a molar ratio of 4:1 in Krebs-Ringer bicarbonate buffer. α-TOH and LCM were dissolved in DMSO. For incubation, the compounds were mixed with supplemented RPMI 1640 medium without serum in the concentrations indicated in the figures.

2.4. Cytotoxicity

THP-1 macrophages were incubated with the respective test compounds in 24-well (standard) or 48-well (transfection) plates; at the end of the incubation period, the cells were washed twice with serum-free supplemented medium. The treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/ml in PBS, 50 μl or 25 μl per well, respectively) in 500 μl or 250 μl serum-free supplemented medium was performed for 4 h. Then the medium was exchanged by 1 ml or 0.5 ml isopropanol and was thoroughly mixed for 10 min, before a centrifugation step (5 min, 300 ×*g*, room temperature) was applied. The solutions were aliquoted (in quadruplicates or triplicates of 100 μl each) to a 96-well plate. Absorption was measured at 570 nm. Viability was calculated by setting the untreated control to 100%. The EC_{50} values for stearic acid under the influence of a defined compound concentration were calculated using a sigmoidal fit for every biological replicate. Only those experiments were included, which revealed a clear EC_{50} value (three out of five biological replicates). EC_{50} values were obtained as whole numbers with no decimal places.

2.5. Concentrations for cell culture studies

The concentrations of the compounds were determined by absorption measurement in pure ethanol. The wavelengths and attenuation coefficients used are 292 nm and $\varepsilon = 3060$ for α-13′-OH and α-13′-COOH.

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Mini kit (Hilden, Germany) as described [24]. cDNA synthesis was performed using Revert Aid First strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and 500 ng/μl oligo-dT primers as described [25].

2.7. Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was run on a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Schwerte, Germany) as described [25,26]. Primers (PLIN2, RPL37A, Supplementary Table S1) were purchased from Invitrogen (Karlsruhe, Germany). PCR results were analyzed using the LightCycler software version 1.5.0.39.

2.8. Transfection

Transfection of THP-1 macrophages was performed as described by Maeß et al. [27] with slight modifications. Cells were differentiated for 24 h using 100 ng/ml PMA. Transfection was performed using $3 * 10^6$ cells, 1% human serum (Sigma-Aldrich), 2 μg siRNA (PLIN2 Stealth siRNA ADFP HSS174700, 5288746 or Stealth RNAi Negative Control Low GC, 12935200; Thermo Fisher Scientific) and mouse T cell nucleofector medium (Lonza, Basel, Switzerland). Transfected cells were seeded in 48-well plates using 200 μl of cultivation medium to prefill wells and 100 μl of cell suspension was added to each well. The incubation with test compounds started 72 h after transfection.

2.9. Immunoblotting

Cells were harvested using a non-denaturing buffer (50 mM Tris-HCl, 0.5% Nonidet P40, 250 mM NaCl, 15 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4) and samples were processed for Western blotting as described earlier [6]. The proteins were separated by SDS-PAGE and transferred to PVDF membrane (VWR, Darmstadt, Germany). Primary antibodies against PLIN2 (mouse anti-ADRP AP125, 1:50) and α-tubulin (mouse anti-α-tubulin clone B-5-1-2, 1:5000) were purchased from PROGEN (Heidelberg, Germany) and Sigma-Aldrich, respectively. Secondary antibodies (rabbit anti-mouse labeled with horseradish peroxidase, 1:5000) from DAKO (Hamburg, Germany) were used. SignalBoost™ Immunoreaction Enhancer Kit (Calbiochem, Darmstadt, Germany) was used for enhancing chemiluminescence signals for PLIN2.

2.10. Flow cytometry to measure neutral lipids via nile red

After incubation, cells were detached by Accutase I treatment (Sigma-Aldrich). Following washing steps with PBS, cells were stained with nile red solution of 1 μg/ml concentration, incubated for 10 min and washed again. A flow cytometric analysis was performed for neutral lipids in a range of 570 to 590 nm.

2.11. Immunofluorescence

Cells were differentiated on glass cover slips in 24-well plates and incubated as described before; cells were fixed with paraformaldehyde and stained using BODIPY 493/503 (20 μg/ml). Cover slips were mounted on slides and a set of ten pictures per cover slide was taken using a fluorescence microscope. Images were transformed to greyscale, inverted and a threshold was set. The size and number of lipid droplets were quantified by Image J using the analyze particles function.

2.12. Extraction of cellular lipids and fatty acid analysis

Cells were differentiated and incubated as described before; cells were scraped in PBS. Total cellular lipids were extracted as described by Dittrich et al. for blood lipids [28]. Due to the small amount of cellular lipids, the thin layer chromatography was skipped, and the gas chromatographic analysis was performed following the FAME preparation.

2.13. Isolation of garcinoic acid and semi-synthesis of α-LCM

Garcinia kola seeds were a gift from AnalytiCon Discovery (Potsdam, Germany). Isolation of garcinoic acid from the African bitter nut *Garcinia kola* and syntheses of the LCM were performed as described [12,29]. Purity of all LCM used was higher than 95%, as confirmed by HPLC-MS.

2.14. Statistics

Data are presented either as means ± standard deviation or as means ± standard error of the mean (SEM) of independent experiments as indicated. In order to test for statistical significance, paired Student's *t*-tests were performed using Microsoft Excel 2010. For the analyses of immunofluorescence data, a nested t-test was performed using R.

3. Results

The LCM of vitamin E emerged as regulatory metabolites with distinct and specific effects [30]. The main goal of the present study was to enlighten another aspect of their biological activity and to strengthen the new perspective on LCM as 'activated' or 'executive' metabolites, similar to the metabolites of vitamin A or D.

It has been reported previously that the LCM modulate foam cell formation in THP-1 macrophages, among others by inducing the expression of CD36 [14]. Using these experiments as a starting point, the present study focused on the regulation of another lipid metabolism-related protein, namely adipophilin (adipose differentiation related protein, ADRP or PLIN2) and the impact of the LCM on saturated fatty acid-induced lipotoxicity, as an important event in lipid-driven diseases [20].

PLIN2 is a lipid droplet-associated protein. Based on studies showing effects of vitamin E on lipid-related proteins and lipid metabolism, we investigated the effect of α-TOH and its metabolites on PLIN2. We found that PLIN2 expression was significantly reduced by α-TOH, after 24 h, mRNA and protein levels decreased by 47% to 26%, respectively ($p < 0.01$; Fig. 1). The LCM however, induced PLIN2 expression on both levels in a range of 1.6- to 3.8-fold ($p < 0.05$). The expression of PLIN2 is known to be induced by fatty acids, such as

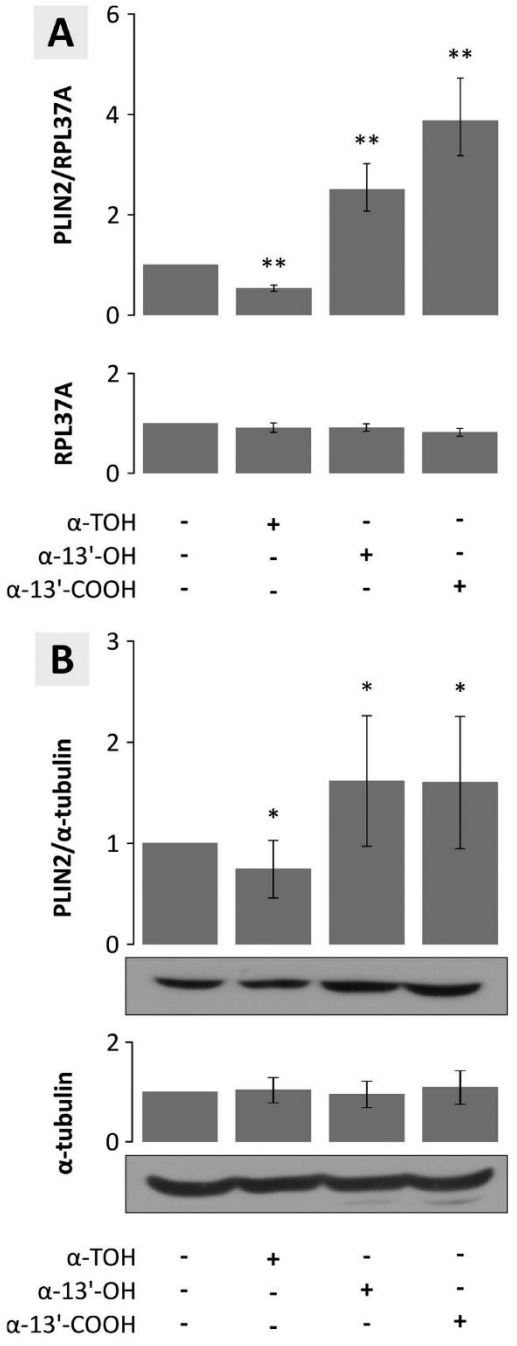

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Fig. 1. PLIN2 expression is induced by α-LCM but reduced by α-TOH.
Human THP-1 macrophages were incubated with 100 μM α-TOH, 10 μM α-13′-OH or 5 μM α-13′-COOH for 24 h and processed for mRNA (A) or protein (B) expression analysis. mRNA expression of PLIN2 was reduced under α-TOH treatment by 47% (SEM min 7%, SEM max 6%) and by 26% ± 28% on protein level. In contrast, the LCM α-13′-OH induced the PLIN2 expression of mRNA level by 250% (SEM min 43%, SEM max 51%) and 162% ± 65% for protein level. More pronounced effects were obvious with α-13′-COOH: an increase of mRNA expression by 388% (SEM min 69%, SEM max 85%) and of protein expression by 160% ± 65% was found. (A) PLIN2 mRNA expression levels were normalized to RPL37A mRNA expression, which remained unchanged under all conditions. Error bars display calculated maximum and minimum expression levels of mean expression levels of three independent biological experiments each measured twice. (B) PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four independent biological experiments each measured twice are shown. *, $p < 0.05$; **, $p < 0.01$ (vs. control).

stearic acid. Hence, we were interested in the regulation of PLIN2 under stearic acid and LCM treatment. Therefore, THP-1 macrophages were pre-treated with α-TOH or the respective LCM for 24 h, followed by a co-incubation with stearic acid for an additional 24 h before protein expression analysis via Western blot (Fig. 2). For both pre-treatments (α-TOH and α-13′-COOH), the co-incubation with stearic acid and LCM resulted in expression values similar to that of stearic acid alone. Similar results were achieved by neutral lipid staining of cells using nile red. The cellular levels of neutral lipids were not influenced by α-TOH but were increased 1.5- to 1.8-fold by α-13′-COOH and stearic acid, respectively. The co-incubation with α-TOH and α-13′-COOH did not affect the stearic acid-induced accumulation of neutral lipids. Furthermore, we analyzed the relative fatty acid composition in THP-1 macrophages, which were incubated with 100 μM α-TOH or 5 μM α-13′-COOH as single compounds or in combination with 150 μM stearic acid for 48 h in total. As expected, significant increase of C18:0 (stearic acid) in the stearic acid treated samples was found (~3.7-fold; $p < 0.001$; data not shown). A very small, but significant difference ($p < 0.01$) in the relative amount of C16:1 (palmitoleic acid) and C18:1 (oleic acid) was observed, when the co-incubated samples were compared (α-TOH vs. stearic acid (3.5% and 21.2%, respectively) or α-13′-COOH vs. stearic acid (3.7% or 21.8%, respectively; data not shown). We also analyzed the size and the number of lipid droplets using BODIPY staining and fluorescence microscopy (Fig. 2E and F). We found an increase in the number of lipid droplets when stearic acid was applied (~3-fold, $p < 0.001$). The size of lipid droplets increased in the presence of α-13′-COOH (~4-fold, at least $p < 0.01$).

Stearic acid is known to induce lipotoxicity, when the concentrations applied exceed the cell's capacity of saturated fatty acid handling. We demonstrated this by incubating the cells with stearic acid in concentrations of up to 800 μM. Thereby, the treatment with a concentration range for α-TOH (0 to 100 μM) or α-13′-COOH (0 μM to 5 μM) made the impact of LCM on lipotoxicity obvious (Fig. 3). While α-TOH worsened the stearic acid-induced lipotoxicity significantly (decrease of EC_{50} value by 139 μM for 50 μM α-TOH), α-13′-COOH partially protected the cells significantly (increase of EC_{50} value by 214 μM for 1 μM α-13′-COOH).

Keeping in mind that the LCM can induce PLIN2, we wondered whether the reduced lipotoxicity of stearic acid by the LCM is mediated via PLIN2. Therefore, knockdown studies of PLIN2 were performed and the impact of its knockdown on stearic acid-induced lipotoxicity was tested. The knockdown was followed by an incubation regime similar to the approach used for Fig. 3 (incubation with 0 μM to 800 μM stearic acid). It was very clear that the cells treated with siRNA against PLIN2 were more prone to the lipotoxic effect of stearic acid than the controls (16% decreased viability by PLIN2 knockdown for 400 μM stearic acid; $p < 0.01$; Fig. 4).

To study the contribution of the PLIN2 knockdown on the protective effect of the LCM on stearic acid-induced lipotoxicity, the transfected cells were pre-incubated with the α-13′-COOH for 24 h and different concentrations of stearic acid were applied for a further 24 h (Fig. 5). The effective stearic acid concentration was calculated for several viabilities (40% to 85%) for both transfections (control siRNA and PLIN2 siRNA) and these were plotted against the respective LCM concentration. The slope for the defined viabilities across the LCM concentration was calculated and these were averaged within the siRNA treatments. A significant difference between these slopes was found (siCTRL: grey line, siPLIN2: dotted line; $p < 0.001$). This means that although a protective effect of α-13′-COOH is still observed under PLIN2 knockdown, it is by far less pronounced than in cells transfected with control siRNA. Therefore, it can be assumed that α-13′-COOH protects from stearic acid-induced lipotoxicity at least partially via the regulation of PLIN2 protein levels.

4. Discussion and conclusions

Recent studies on the metabolism of vitamin E revealed the physiological presence of the LCM α-13′-OH and α-13′-COOH in human blood [7,14]. We therefore hypothesize that the LCM are available at the site of action, e.g. in the case of atherogenesis at intimal macrophages. A recent study provided convincing evidence for the specific and distinct signaling mediated by LCM even at concentrations lower than their precursors [30]. Based on these results, a specific, not yet identified receptor for LCM was proposed. To elucidate the biological and molecular mechanisms of the LCM in more detail, a study on the regulatory effect of the LCM on the scavenger receptor CD36 [14] was used as a starting point for the experiments described here. We aimed to get a deeper insight into the regulation of foam cell formation by the LCM and focused on the regulation of PLIN2 at basal conditions and under load with the saturated stearic acid. Therefore, we used the LCM and α-TOH in concentrations which have been already used in other studies [7,14,31,32].

The expression of the lipid droplet-associated protein PLIN2 was assessed in human THP-1 macrophages after α-TOH and α-LCM treatment under basal conditions, i.e. incubation without serum. To the best of our knowledge, we are the first to describe the inhibition of PLIN2 expression by α-TOH and the induction of PLIN2 mRNA and protein level by the α-LCM. The neutral lipid accumulation precisely followed the regulation of PLIN2, as measured after incubation of 48 h by nile red staining and subsequent flow cytometric analysis. Due to delayed accumulation of neutral lipids (no significant increase in lipid levels were observed after 24 h (data not shown), but prominent accumulation was found after 48 h; Fig. 2), we conclude that the LCM induce PLIN2 expression, which in turn leads to lipid accumulation. This is plausible since PLIN2 is known to elevate cellular lipid levels by inhibiting β-oxidation [33] and lipolysis [34]. Thus, the lipid storage capacity of the cells is increased by the LCM via induced PLIN2 expression, followed by increases in neutral lipid accumulation. In a recent study, Bartolini et al. treated HepG2 cells with α-TOH and found an increase in lipid accumulation (Oil Red O staining) [35]. Unfortunately, no experiments using the LCM have been reported in this context.

In contrast to the aforementioned experiments, we focused on experiments under fatty acid stimuli. While stearic acid incubation led to the expected increase in PLIN2 protein expression [36] on protein level and in neutral lipid accumulation [37], the co-incubation with stearic acid and α-13′-COOH showed no additive effect (Fig. 2), which was also reflected by the gas chromatographic analysis of the cells (data not shown). This can be explained by mechanisms each specific for LCM and stearic acid. The readout (PLIN2 expression and lipid accumulation) may lead to the same results, but the underlying mechanisms are likely to be distinct. For stearic acid, a direct interaction with the PLIN2 protein via specific binding pockets has been described [38], while

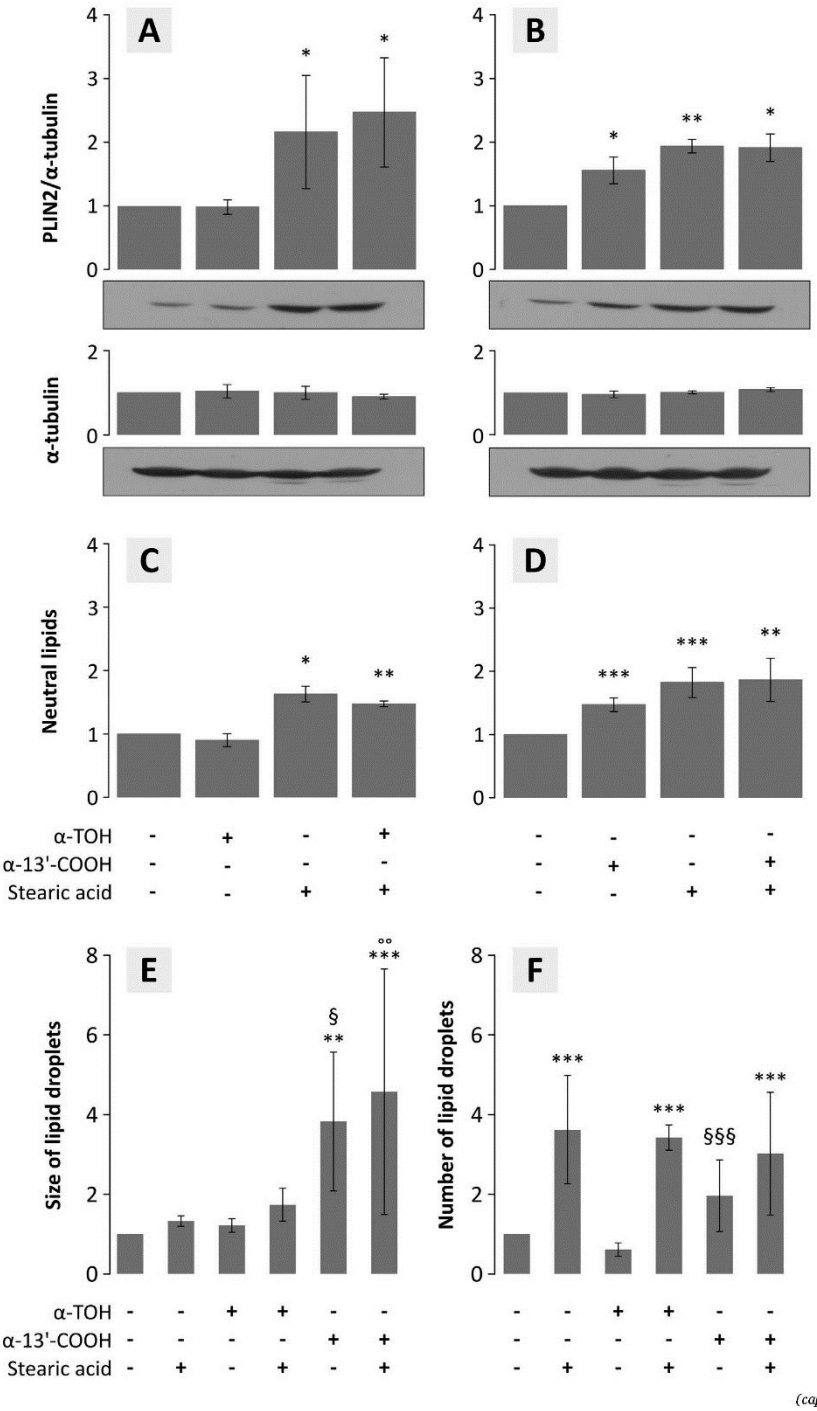

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Fig. 2. PLIN2 expression and neutral lipid accumulation is induced by stearic acid and α-13′-COOH.
Human THP-1 macrophages were incubated with 100 μM α-TOH (A, C, E, F) or 5 μM α-13′-COOH (B, E, F) or 2.5 μM α-13′-COOH (D) for 24 h and with 150 μM stearic acid for an additional 24 h in the presence or absence of the compounds. Afterwards, cells were processed for protein expression analysis via Western blot (A + B), neutral lipid staining (C + D) or immunofluorescence (E + F).
(A) While α-TOH did not regulate PLIN2 protein expression, stearic acid-induced PLIN2 by 216% ± 66% and the combinatory incubation resulted in an induction of 222% ± 67%. (B) The LCM α-13′-COOH increased the PLIN2 protein expression by 156% ± 21%, while stearic acid upregulated PLIN2 expression by 194% ± 11%. In combination, both compounds increased PLIN2 protein expression by 191% ± 22%. PLIN2 protein expression levels were normalized to α-tubulin expression, which remained unchanged under all conditions. Images of Western blot analyses show representative results. Mean expression levels of four (A) or three (B) independent biological experiments each measured once are shown.
(C) While α-TOH did not regulate neutral lipid accumulation, stearic acid induced neutral lipids by 163% ± 13% and the combinatory incubation resulted in an induction of 147% ± 5%. (D) α-13′-COOH induced neutral lipid accumulation by 147% ± 11%, while stearic acid-induced neutral lipid accumulation by 182% ± 24%. In combination, both compounds induced neutral lipid accumulation by 186% ± 34%. Neutral lipid accumulation was calculated relative to the control set to 1. Mean expression levels of three (C) or five (D) independent biological experiments each measured once are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs. control).
(E) The size of lipid droplets was increased by α-13′-COOH by 383% ± 174% or by α-13′-COOH and stearic acid by 457% ± 308%. (F) The number of lipid droplets was increased by stearic acid (362% ± 136% for stearic acid, 342% ± 32% for stearic acid and α-TOH, 302% ± 154% for stearic acid and α-13′-COOH). Size and number of lipid droplets were calculated relative to the control which was set to 1. Three biological replicates were prepared and a set of ten pictures per sample was taken. The data were evaluated using a nested *t*-test. p-Values are only shown for biologically relevant comparisons: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (vs. control); § vs. α-TOH; ° vs. stearic acid.

analogous studies are pending for the LCM. However, it is not yet established whether this influences neutral lipid accumulation or PLIN2 expression. Each mechanism may also be regulated or influenced by the respective other compound, which in turn may lead to the non-additive effect seen under the co-treatment setup. Another important aspect might be the heterogeneity of lipid droplets [39,40], which could be influenced by the LCM. Lipid droplets vary not only in size or contact sites to other organelles, e.g. ER [41], but also in lipid [40] and protein [42] composition. Interestingly, PLIN2 is localized on lipid droplets of all sizes (starting with pre-lipid droplets) [41,42]. We also report here that the LCM induce the mean size of intracellular lipid droplets. This may contribute to the protective effect of the LCM in the lipotoxic setup described here.

We focused on the interaction of α-TOH and LCM with stearic acid-induced lipotoxicity. We speculated that the LCM may induce a conversion of saturated fatty acids to monounsaturated fatty acids (e.g. C16:0 to 16:1 or C18:0 to C18:1). This has been considered to reduce lipotoxicity of saturated fatty acids [43]. Since the gain of viability by the conversion of C18:0 to C18:1 is higher than for C16:0 to C16:1 [44], we decided to use stearic acid (C18:0) for lipotoxicity experiments in order to reduce the compensatory effect of the conversion. For this purpose, cells were treated with a compound-concentration-matrix of stearic acid (0 to 800 μM) and α-TOH (0 to 100 μM) or α-13′-COOH (0 to 5 μM). Stearic acid-induced lipotoxicity, measured by the MTT viability test, has been investigated [43]. Here, we describe for the first time that stearic acid-induced lipotoxicity was worsened by α-TOH, whereas α-13′-COOH reduced the effect of stearic acid. So far, neither the effects of vitamin E nor its metabolites on lipotoxicity have been investigated in detail, thus the underlying mechanisms are an object of speculation. Besides the regulatory induction of PLIN2, the inhibition of apoptosis via unknown mechanisms may also contribute to the inhibition of lipotoxicity. Results from an analogous study designed by Rabkin et al. [43] were striking. Rabkin also induced lipotoxicity via stearic acid, but oleic acid (C18:1) was used for rescuing cardiomyocytes from death. Surprisingly, the intracellular lipid pattern measured under co-incubation of stearic acid and oleic acid was similar to our results for α-13′-COOH in neutral lipid accumulation, which is a non-additive effect. Keeping in mind the low similarity in their structure, the similar findings for the LCM and oleic acid are unexpected.

We would like to point the reader's attention to the impact of fatty acid distribution of PLIN2 binding on the surface and size of lipid droplets. The binding affinity of PLIN2 for oleic acid is twice that of stearic acid [45], and introducing saturated acyl chains to the phospholipids of the monolayer forming the surface of lipid droplets impairs PLIN2 binding due to the condensation of the phospholipid monolayer covering lipid droplets [46]. However, larger intracellular lipid droplets have a higher proportion of saturated fatty acids in their monolayer than smaller ones [47]. Unfortunately, fatty acid profile of cells treated with 100 μM α-TOH, 5 μM α-13′-COOH or 150 μM stearic acid or the combination of vitamin E derivate and stearic acid did not complete the puzzle. The most obvious finding was the relative increase of stearic acid, when stearic acid was added to the cells. Under treatment with stearic acid, α-13′-COOH also minimally increased the relative amount of the monounsaturated form of C16:0 (C16:1, +0.2%) and C18:0 (C18:1, +0.6%) compared to the α-TOH treated samples. It is known that the monounsaturated fatty acids are less toxic than the saturated forms [44]. This might explain the improved viability of cells treated with α-13′-COOH to some extent. However, keeping the effect sizes in mind, one must admit that it is most likely not a single mechanism that prevents the cells from lipotoxicity.

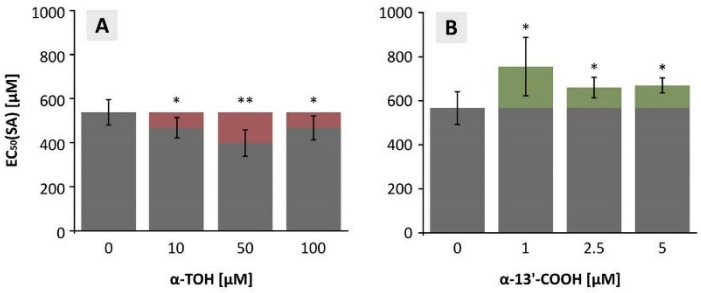

Fig. 3. Stearic acid-induced lipotoxicity is partially reduced by α-13′-COOH but not by α-TOH.
Human THP-1 macrophages were incubated with 0 to 100 μM α-TOH (A) or 0 to 2.5 μM α-13′-COOH (B) for 24 h, followed by a co-incubation with 0 to 800 μM stearic acid for further 24 h. This incubation matrix was assessed by MTT cytotoxicity tests. (A) Treatment of THP-1 macrophages with increasing concentrations of stearic acid resulted in reduced cell viability. The same holds true for cells which were co-treated with α-TOH; here, α-TOH decreased stearic acid-induced lipotoxicity significantly (decrease of EC_{50} value by 70–139 μM) $p < 0.05$ to $p < 0.01$). (B) Again, the concentration-dependent reduction of viability under stearic acid treatment was seen. When cells were co-incubated with α-13′-COOH the viability of the cells was significantly increased (increase of EC_{50} value by 91–214 μM $p < 0.05$). *, $p < 0.05$; **, $p < 0.01$; (vs. control).

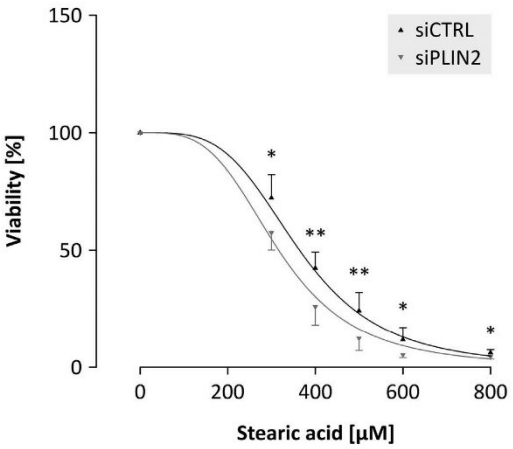

Fig. 4. PLIN2 partially protects from stearic acid-induced lipotoxicity.
Human THP-1 macrophages were transfected with control siRNA (siCTRL) or PLIN2 siRNA (siPLIN2) and incubated with increasing concentrations of stearic acid (0 to 800 µM) for 24 h. Afterwards, the cells were treated with MTT solution and the readout was performed as described in the Materials and Methods section. With increasing concentrations of stearic acid, the viability of control cells decreased (300 µM stearic acid: 72.26% ± 9.9% viability, 500 µM stearic acid: 24.21% ± 5.1% viability). Cells treated with PLIN2 siRNA were more prone to stearic acid-induced lipotoxicity (300 µM stearic acid: 57.47% ± 7.4% viability, $p < 0.05$ (control vs. PLIN2 siRNA treatment), 500 µM stearic acid: 12.39% ± 5.3% viability, $p < 0.01$ (control vs. PLIN2 siRNA treatment)). Error bars display standard deviations of mean viability levels of four independent biological experiments each measured once. *, $p < 0.05$; **, $p < 0.01$ (vs. siCRTL).

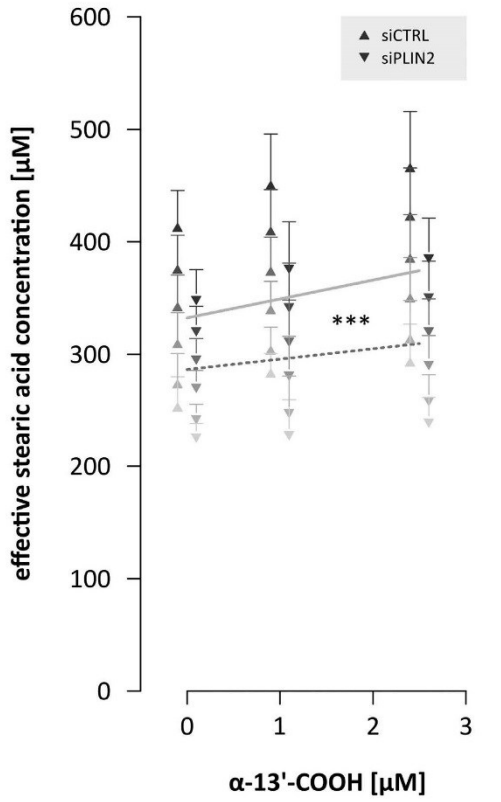

Fig. 5. α-13′-COOH partially protects from stearic acid-induced lipotoxicity via PLIN2.
Human THP-1 macrophages were transfected with control siRNA (siCRTL) and PLIN2 siRNA (siPLIN2) and incubated with 0 to 2.5 µM α-13′-COOH and 0 to 800 µM stearic acid. A plot showing the dependency of the viability from stearic acid concentration was calculated for each LCM concentration. A logarithmic fit was performed, and the effective concentrations of stearic acid for 40% to 85% viability were calculated. For each viability, the slope for the concentration dependency of LCMs on the effective concentration of stearic acid was calculated. The mean for each effective concentration of stearic acid was obtained from four biological replicates. Finally, the mean was calculated across all experiments, which were treated with the same siRNA. The comparison of both slopes, siCRTL (grey line) vs. siPLIN2 (dotted line) dependent on LCM concentration, and the effective concentration of stearic acid revealed a significant difference (***, $p < 0.001$).

The effect of α-TOH or LCM on stearic acid-induced lipotoxicity is accompanied with the regulation of PLIN2 expression. Therefore, we asked whether PLIN2 might be the crux of the matter and thus set up knockdown experiments for PLIN2. At basal conditions (stearic acid concentration gradient only, no incubation with α-13′-COOH), a worsening of stearic acid-induced lipotoxicity was found, which is likely due to a loss in lipid storage capacity forced by the knockdown of PLIN2. Knockdown of PLIN2 has been described as influencing cellular lipid levels and size of lipid droplets as well as their number [33,48]. It has also been suggested that PLIN2 plays a role in the expansion of lipid droplet size [49]. In summary, this could lead to the disruption of stearic acid-induced lipotoxicity by the knockdown of PLIN2. However, it has been described that other homologs of PLIN2, e.g. TIP47 [50] take on its task if PLIN2 is knocked down. This might be the reason why the decrease in stearic acid-induced lipotoxicity by the knockdown of PLIN2 is relatively mild. Taken together, PLIN2 is protective against stearic acid-induced lipotoxicity to some extent.

A cross-comparison of the respective cell viabilities between Figs. 3 and 4 highlights the increased sensitivity of transfected cells towards stearic acid treatment. It is known that electroporation (the transfection method used here) is able to transfer lipids between membrane leaflets [51], which may induce further perturbations in lipid metabolism or handling. Overall, this might contribute to the higher sensitivity of transfected cells to mediators of lipotoxicity.

Next, the stearic acid lipotoxicity tests were conducted in the presence of the knockdown of PLIN2 and LCM incubation. While the LCM-dependent blocking of lipotoxicity was observed under both conditions (control and PLIN2 knockdown), the effective stearic acid concentrations needed to achieve a certain viability, were significantly reduced by the knockdown of PLIN2 across all α-13′-COOH concentrations tested. This leads to two conclusions: (i) PLIN2 expression protects macrophages from stearic acid-induced lipotoxicity, and (ii) the LCM partially protect macrophages from lipotoxicity via the induction of PLIN2. It might be striking that the reduction in lipotoxicity by α-13′-COOH seems to not be coupled with an additive induction of neutral lipid accumulation. On the other hand, it has been shown that the cellular lipid content is not a determinant of lipotoxicity-induced cell death [43].

The underlying signaling pathways, which are responsible for the distinct LCM effects are currently the focus of our research. Our latest study indicated that a molecular receptor for the LCM might exist [30]. However, our most recent unpublished findings revealed insights into more complex molecular interactions, which require a comprehensive

evaluation that is ongoing.

Overall, we have made several new observations: First, PLIN2 protects macrophages from stearic acid-induced lipotoxicity. Second, α-TOH triggers stearic acid-induced lipotoxicity in macrophages. Third, we found evidence for a complex link between the α-LCM, PLIN2 and lipotoxicity. We provide first evidence for the potentially protective effects of the α-LCM by inducing lipid storage capacity and thereby decreasing the proneness to lipotoxicity. Our data also show that PLIN2 contributes at least partially to this phenomenon. Our results will contribute to the growing knowledge on the modes of action of the LCM, which we consider as a new class of regulatory metabolites.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2018.05.002.

Conflict of interest statement

The authors declare no competing interests.

Transparency document

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7.4 Manuscript IV

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Research Paper

The vitamin E derivative garcinoic acid from *Garcinia kola* nut seeds attenuates the inflammatory response

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ABSTRACT

The plant *Garcinia kola* is used in African ethno-medicine to treat various oxidation- and inflammation-related diseases but its bioactive compounds are not well characterized. Garcinoic acid (GA) is one of the few phytochemicals that have been isolated from *Garcinia kola*.

We investigated the anti-inflammatory potential of the methanol extract of *Garcinia* kola seeds (NE) and purified GA, as a major phytochemical in these seeds, in lipopolysaccharide (LPS)-activated mouse RAW264.7 macrophages and its anti-atherosclerotic potential in high fat diet fed ApoE $^{-/-}$ mice.

This study outlines an optimized procedure for the extraction and purification of GA from *Garcinia kola* seeds with an increased yield and a purity of >99%. We found that LPS-induced upregulation of iNos and Cox2 expression, and the formation of the respective signaling molecules nitric oxide and prostanoids, were significantly diminished by both the NE and GA. In addition, GA treatment in mice decreased intra-plaque inflammation by attenuating nitrotyrosinylation. Further, modulation of lymphocyte sub-populations in blood and spleen have been detected, showing immune regulative properties of GA.

Our study provides molecular insights into the anti-inflammatory activities of *Garcinia* kola and reveals GA as promising natural lead for the development of multi-target drugs to treat inflammation-driven diseases.

1. Introduction

Natural products obtained from plants are widely used in folk medicine. The number of novel natural products described every year is large and systematic efforts are needed to elucidate their effectiveness and functions as bioactive principles or lead structures for drug development. A good example for the use of extracts in phytomedicine is the African plant *Garcinia kola* [1], which was first described for its antimicrobial properties by Hussain et al., in 1982 [2]. Until today several additional effects, such as radical scavenging [3], anti-oxidative [4] and anti-inflammatory properties [5], have been reported. Since this plant contains several bioactive compounds, namely garcinoic acid (GA) [6], it represents an interesting source to study putative pharmacological actions [7].

Our compound of interest, GA, also known as *trans*-13′-carboxy-δ-tocotrienol, contains an oxidative modification at its side chain and is a principle hepatic metabolite of dietary δ-tocotrienol (T3) [6]. In addition to tocopherols (TOH), T3s represent a less abundant form of vitamin E. TOHs and T3s, which differ in the saturation of the side chain, are further divided into α-, β-, γ- and δ-forms showing specific methylation patterns of the chromanol ring. However, in the last decades T3s have been described to potently interfere with inflammation and

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oxidative stress *in vitro* [8,9] and in animal models [10,11]. In addition, T3s affect macrophage recruitment [12] – a key event in atherosclerosis. In line with this data, anti-atherosclerotic effects of T3s have been shown in ApoE$^{-/-}$ mice by Shibata and colleagues [13].

Recent studies demonstrated that carboxylation of the side chain significantly increases the anti-inflammatory capacity of TOHs [14–16]. Similar effects have been demonstrated for GA, an oxidized δ-T3, which inhibits mPGES-1 *in vitro* [17]. Therefore, we investigated the anti-inflammatory effects of GA in comparison to the methanol extract of *Garcinia kola* seeds (NE) in LPS-activated RAW264.7 macrophages to elucidate the contribution of the latter phytochemical. Further, we studied the effectiveness of GA in decreasing inflammation-related formation of atherosclerotic plaques using an atherosclerotic mouse model to estimate the potential of GA as a promising new therapeutic lead molecule against inflammation-driven diseases.

2. Materials and Methods

2.1. Chemicals

If not indicated otherwise, chemicals were obtained from Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Seelze, Germany), or Merck Millipore (Darmstadt, Germany).

2.2. Extraction of Garcinia kola seeds and isolation of GA

2.2.1. Standard preparation of NE

The standard preparation of the NE was performed according to published procedures [4,18,19] (Suppl. Fig. S1).

2.2.2. Optimized preparation of NE

NE from *Garcinia kola* seeds was obtained using Bligh and Dyer extraction [20]. Thus, 100 g crushed seeds and methanol/chloroform (400 ml/800 ml) were shaken for 4 h. After filtering, 400 ml of a 2% (w/v) NaCl solution was added and the mixture was shaken vigorously for 5 min. The chloroform phase was dried using Na_2SO_4 and the solvent was evaporated (Suppl. Fig. S1).

2.2.3. Isolation and purification of GA

Purification of GA was performed as reported with slight modifications [4,18,19]. In brief, seed extract was dissolved in methanol/chloroform (95%/5%, v/v) and applied to a silica gel column to isolate a crude product. Presence of GA in collected eluates was tested using thin-layer chromatography with dichloromethane/methanol (95%/5%, v/v) as solvent. Subsequently, re-chromatography of GA-containing aliquots was performed on a silica gel using a hexane/acetone (65%/35%, v/v) mixture. GA was characterized by high-performance liquid chromatography coupled with mass spectrometry (Fig. 1 and flow chart in Suppl. Fig. S1).

2.2.4. Liquid chromatography coupled with tandem MS (LC-MS/MS) analysis

The LC-MS/MS system consisted of a Dionex UltiMate 3000 UHPLC system coupled to a Bruker AmaZon SL Ion trap mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) source (Bruker, Karlsruhe, Germany). The chromatography utilized a Kinetex F5 Core-Shell column (2.1 × 100 mm, 2.6 µm) from Phenomenex (Aschaffenburg, Germany) connected to a SecurityGuard ULTRA cartridge (Phenomenex). The solvent system consisted of methanol/formic acid (1000:1 v/v, A) and H_2O/formic acid (1000:1 v/v, B). The separation was performed with a multi-step gradient scheme as follows: 0 min, 70% B; 3 min, 70% B; 5 min, 80% B; 10 min 80% B; 12 min, 90% B; 18 min, 90% B; 20 min, 100% B (flow rate 0.2 ml/min). Data were analyzed using basic peak monitoring and negative polarity APCI (dry gas temperature: 250 °C, flow: 4.2 l/min; nebulizer pressure: 34.8 psi; vaporizer temperature: 380 °C; capillary voltage: 4000 V; end plate offset: 500 V) with Bruker Compass Data Analysis software version 4.2.

2.3. RAW264.7 macrophage culture

Murine RAW264.7 macrophages (ATCC, Manassas, VA) were cultivated as described previously [16]. For experiments, cells were incubated as indicated in the figure legends and harvested for further processing as described below. For detailed information, see the Suppl. Materials and Methods section.

2.4. Animal experiments and treatment

All animal procedures were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia (Ethic number E/1658/2016/B) and was performed in accordance with the Australian code for care. To investigate the effect of GA on the progression of atherosclerosis, eight weeks old male apolipoprotein E knockout (ApoE$^{-/-}$) mice (C57Bl/6 background, 25–28 g), ad libitum fed a HFD (22% fat and 0.15% cholesterol, SF00-219, Specialty Feeds, Western Australia, Suppl. Table S1) for further eight weeks, have been used. Mice were randomly assigned to receive 1 mg/kg body weight of GA (n = 9) or vehicle (PBS + 0.8% DMSO, n = 9) via intraperitoneal (IP) injection weekly. At the age of 16 weeks, mice have been anesthetized using ketamine (50 mg/kg, Parnell Laboratories, NSW, Australia) and xylazine (10 mg/kg, Troy Laboratories, NSW, Australia). Blood and tissue samples were collected and processed as described below. Grouping of animals and quantifications were blinded from the responsible researchers throughout the study.

2.5. RNA isolation, cDNA synthesis and quantitative real-time PCR (RT-qPCR)

Total RNA isolation (Qiagen, Hilden, Germany), cDNA synthesis (Fermentas, St. Leon-Rot, Germany) and RT-qPCR analysis (LightCycler 480 II instrument, Roche Diagnostics, Mannheim, Germany) have been performed as described previously [16]. Primers (Suppl. Table S2) were purchased from Invitrogen (Karlsruhe, Germany).

2.6. Immunoblotting

Cell harvesting, sample preparation and antibody usage are according to Wallert et al. [16] For the detection of α-tubulin (55 kDa), Cox2 (72 kDa) and iNOS (130 kDa), PageRuler™ Prestained Protein Ladder (10–180 kDa) from Thermo Fisher Scientific (Schwerte, Germany) was used.

2.7. Quantification of nitric oxide (NO) formation using Griess assay

Griess assay was used to measure nitrite in the supernatant of RAW264.7 macrophages (Enzo Life Science) and in murine plasma samples (Promega). Macrophages were incubated with either solvent (DMSO), 1.25 µg/ml NE or 2.5 µM GA in serum free high glucose DMEM for 4 h followed by a combined incubation with 100 ng/ml LPS for 20 h. For Griess assay, collected cell supernatants were prepared as outlined in Wallert et al. [16]. Murine plasma samples were prepared according to manufacturer's protocol. Absorbance was measured at 540 nm using a Fluostar omega plate reader (BMG Labtech, Offenbach, Germany and Adelaide, Australia).

2.8. Quantification of prostanoids using reversed phase UPLC-MS/MS and ELISA

Thromboxane (Tx)B_2 release from RAW264.7 macrophages was measured using an ELISA Kit from Enzo life Sciences (Lörrach, Germany) according to the manufacturer's protocol. In addition,

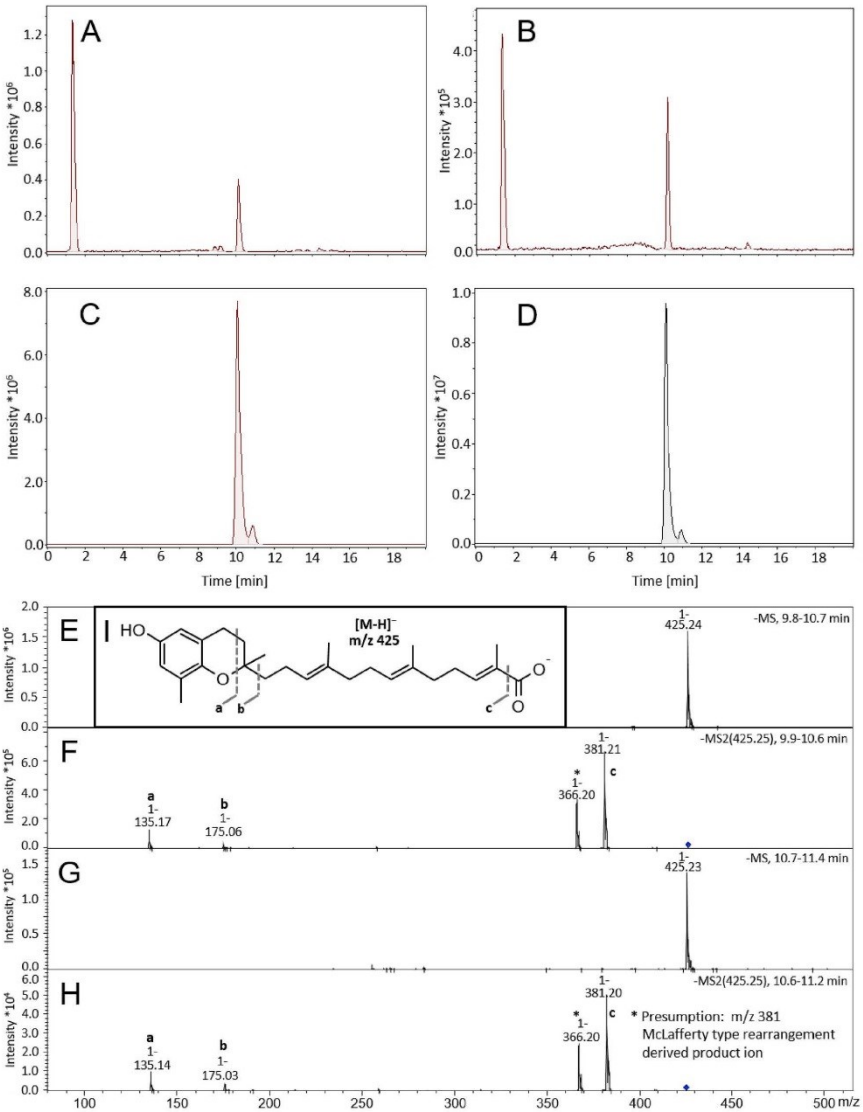

Fig. 1. Bligh and Dyer extraction increased the yield of garcinoic acid (GA) isolated from *Garcinia kola* seeds at high purity. Representative LC-MS chromatograms of the *Garcinia kola* seeds extracts obtained by the standard procedure **(A)** and by Bligh and Dyer extraction **(B)**. Panel **(C)** and **(D)** show LC-MS chromatograms of the purified GA obtained from crude methanol extract from *Garcinia kola* seeds according to the procedures used for **(A)** and **(B)**, respectively. Mass spectra of the purified GA were obtained from the LC-MS chromatogram **(D)** for two peaks with retention times of 9.8–10.6 min (main peak, **E**) and 10.7–11.4 min (minor peak, **G**). MS/MS fragmentation spectra of **(E)** and **(G)** are shown in panels **(F)** and **(H)**. The fragmentation is indicated on structure **(I)**, respectively.

prostaglandins and TxB_2 were extracted from RAW264.7 cell supernatants and murine plasma, separated on an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters, Milford, MA) using an Acquity™ UPLC system (Waters), and detected using a QTRAP 5500 MS (Sciex, Darmstadt, Germany) equipped with an electrospray ionisation source as previously described [16].

2.9. Histology and immunohistochemistry

For histology and immunohistology transversal cryosections of O.C.T. embedded aortic sinus (6 µm) were prepared (Zeiss MICROM HM 550). For detailed information see Suppl. Materials and Methods section.

2.9.1. Histological staining

For H&E staining, fixed sections (70% EtOH, 10% formalin, 5% glacial acetic acid, 15% dH_2O, 5 min) were washed twice in 70% EtOH (30 s) and dH_2O (5 min) and stained with Mayer's Hematoxylin (2 min), followed by Puff's Eosin solution (20 s), dehydrated and cleaned using 100% EtOH and Xylene, respectively. Finally, samples were mounted with Depex to ensure their longevity. To stain collagen content, samples were fixed in 10% formalin for 20 min and placed in 0.1% PSR (0.5 g Sirius Red in 500 ml Picric Acid Solution) solution for 1 h. After differentiation with 0.01 M HCl, samples were washed, dehydrated, cleaned and mounted as described above. Lipid content was determined using isopropanol/dH_2O diluted (3:2 v/v) and filtered ORO. Samples were fixed (10% formalin, 4 min) and washed in 60% isopropanol (25 s) before staining with ORO (1 h), differentiated in 60% isopropanol, washed, counterstained with Mayer's hematoxylin (45 s) and mounted in Aquatex (Merck Millipore, Bayswater, VIC, Australia).

2.9.2. Immunohistochemical staining

Quantification of plaque stability, intra-plaque inflammation and cell infiltration was determined using antibodies against nitrotyrosine, Interleukin (IL)1β, vascular cell adhesion protein (VCAM)-1, monocyte chemoattractant protein (MCP)-1 and the macrophage marker CD68. Fixed sections were washed with PBS, blocked with 3% hydrogen peroxide, washed with PBS supplemented with 0.05% Tween20 and blocked with 10% horse or rabbit serum according to host of antibody species. After Avidin and Biotin blocking (Vector Laboratories, Burlingame, CA, USA), except for IL1β and MCP-1 staining, primary antibodies, diluted in blocking solution, were applied. Afterwards, washed samples were incubated with the respective secondary antibody diluted in blocking solution for 30 min, followed by conversion of the chromophoric horseradish peroxidase substrate diaminobenzidine (Vector Laboratories). Finally, samples were counterstained, dehydrated with 95% EtOH followed by 100% EtOH (twice), cleaned using Xylene (twice) and mounted with Depex. Respective IgG and omit antibody controls have been performed (Suppl. Fig. S2). Positive stained area of four sections per sample were analyzed using OPTIMAS version 6.2 VideoPro-32 system.

2.10. Flow cytometer analysis to investigate immunologic cell pattern

Antibodies were purchased from BD Bioscience if not otherwise indicated. Blood was collected in 0.5 M anti-coagulant ethylenediaminetetraacetic acid (EDTA) using cardiac puncture. Spleen was removed and stored on ice (PBS, 2 mM EDTA, 0.1% bovine serum albumin, BSA). Within 1 h of collection, cells from blood and spleen have been isolated, red blood cells were lysed (BD FACS lysing solution), and remaining cells were filtered and plated for further staining. Total monocyte/macrophage population was detected using CD11b-FITC and CD115-PE-Cy7 antibodies (Biolegend, San Diego, CA, USA). In addition, Ly6C-PB staining was performed to separate pro- and anti-inflammatory monocytes sub-populations. B cell lymphocytes have been gated using CD19-PE (BD Bioscience) staining. T cell lymphocytes were categorized in CD4 (PB) and CD8 (PerCP) positive cells as well as natural killer T (NKT) cells via NK1.1-PE-Cy7 and T-cell receptor β (TCR-β) staining. NK1.1-PE-Cy7 positive and TCR-β negative cells were gated as natural killer (NK) cells (Suppl. Fig. S3). Cell populations were analyzed using flow cytometry (FACSCanto II, BD Biosciences, USA) and analyzed with BD FACS DIVA software version 8.0.1.

2.11. Lipid measurement

Blood samples were taken as described above and centrifuged (300×*g*, 10 min, room temperature) to separate plasma within 1 h of collection. Total serum cholesterol, LDL, HDL and triglycerides were measured using COBAS Integra 400 Plus blood chemistry analyzer (Roche Diagnostics, Australia).

2.12. Statistics

Data are presented either as means ± standard deviation (SD) or as means ± standard error of the mean (SEM) of independent experiments. In order to test for statistical significance, paired Student's t-tests was performed using Microsoft Excel 2010. ANOVA followed by Tukey post-hoc tests with logarithmized values and one-way ANOVA with multiple comparisons were used as outlined in the respective figure legends.

3. Results

In the here outlined study we pursued two aims. Firstly, the optimization of the extraction procedure of GA starting from *Garcinia kola* seeds to ensure the most effective use of these rare seeds. Secondly, the characterization of GA, as one of the main phytochemicals in *Garcinia kola* seeds, by focusing on anti-inflammatory effects and its potential as a drug for the treatment of inflammation-driven diseases.

3.1. Bligh and Dyer extraction increases the yield of GA

The extraction method of GA from *Garcinia kola* seeds firstly published by Terashima et al. [4] and slightly modified by Birringer et al. yields up to 0.38% GA[18]. The isolation of the GA follows in principle two steps: (i) the methanol extraction of the seed, and (ii) the purification of GA. Using our optimized approach, we were able to increase the quantity of NE 2.3-fold and the purified GA 6.6-fold (Suppl. Fig. S1) and were able to highly purify GA by silica gel chromatography (>99%). Chromatographic separation revealed a major peak with *m/z* 425.4 $[M\text{-}H^+]^-$ at a retention time (RT) of 10.2 min representing GA and a minor peak at a RT of 10.8 min with identical mass. As the MS/MS spectra of the two peaks showed identical fragmentation patterns, we assume that the minor compound is a stereoisomer (either the *cis/trans* or the diastereomeric form) of GA (Fig. 1).

3.2. Purified GA blocks the LPS-induced expression of inflammatory mediators in murine macrophages

Previous studies demonstrated anti-inflammatory potential of a *Garcinia kola* seeds extract in rats [21]. One of the major compounds in this extract is GA, a T3 metabolite [18]. It has been shown that T3s are highly potent in blocking the inflammatory response of macrophages [8]. Therefore, we investigated the effects of both the NE and purified GA on LPS-induced inflammatory response in murine RAW264.7 macrophages. First, we analyzed the effect of GA on the expression of classic LPS-responsive genes such as Il6, Il1β, Tnfα, Cox2 and iNos, which encode pro-inflammatory mediators (Suppl. Table S2). Neither the NE nor GA affected basal expression levels of these marker genes (Fig. 2A–F, white bars). As expected, LPS significantly induced the expression of the genes of interest. The LPS response was efficiently blocked by both the NE and even stronger by GA as indicated in the figures (Fig. 2A–F, grey bars). Expression of Tnfα was significantly blocked to similar extent by different concentrations of GA (Fig. 2A, right column), whereas the NE tended to decrease Tnfα expression (Fig. 2A, left column). GA blocked the LPS-induced expression of LPS-responsive genes Il6, Il1β, Cox2 and iNos dose-dependently in concentrations of 1, 2, and 5 μM. At a concentration of 5 μM, GA significantly decreased Il6, Il1β, Cox2 and iNos RNA expression to 30% ($p < 0.05$), 39% ($p < 0.001$), 30% ($p < 0.05$) and 3% ($p < 0.05$), respectively (Fig. 2B–E, right column).

3.3. NE and GA differently affect the LPS-induced upregulation of iNos and Cox2 protein expression and secretion of respective signaling molecules

Further, we investigated the effect of NE and GA on post-translational expression of iNos and Cox2. In non-stimulated RAW264.7

macrophages, protein levels of iNos and Cox2 were neither detectable nor modulated by GA or NE. Treatment with NE and GA decreased LPS-induced protein expression of Cox2 to 62% ± 23% (left) and 67% ± 13% (right, $p < 0.0001$; Fig. 3A), respectively. The iNos protein expression was significantly diminished by NE (68% ± 10%, left) and GA (17% ± 11%, right, Fig. 3B, $p < 0.0001$) to a similar extent. Since

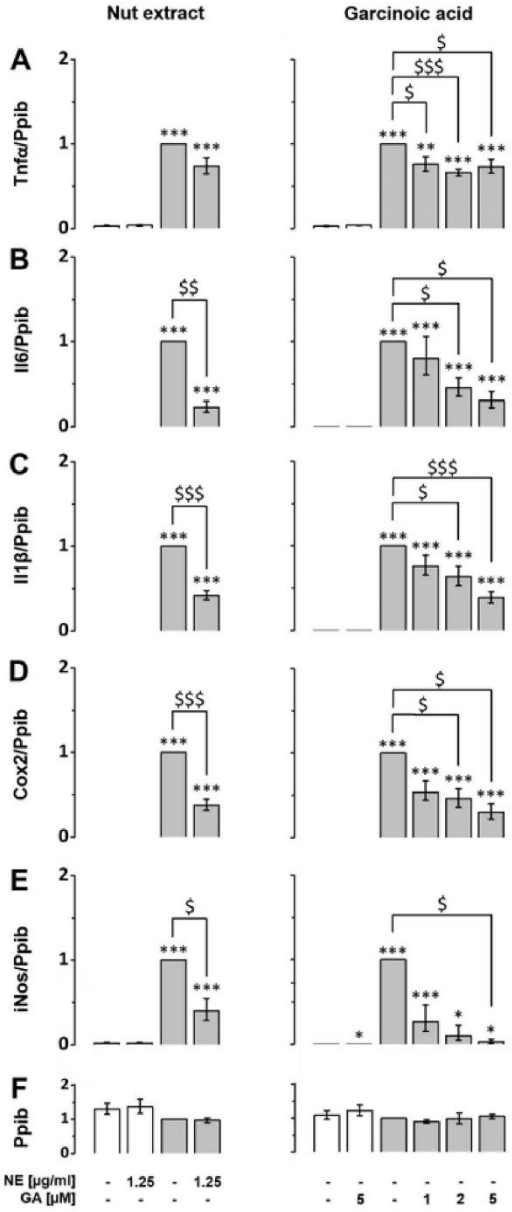

Fig. 2. Lipopolysaccharide-induced upregulation of Il6, Il1β, Cox2, iNos and Tnfα mRNA expression is blocked by the NE and isolated GA. RAW264.7 were pre-incubated with either NE (left column) GA (right column) or solvent (DMSO) for 24 h (white bars). For LPS-induced experiments, macrophages were co-incubated with 100 ng/ml LPS and either solvent, NE or GA at the doses indicated for another 24 h (grey bars). Samples co-cultured with solvent and LPS were defined as one. Expression levels of the inflammatory response genes **(A)** Tnfα, **(B)** Il6, **(C)** Il1β, **(D)** Cox2 and **(E)** iNos were measured using RT-qPCR and normalized to the mRNA expression of the reference gene **(F)** peptidylprolyl isomerase B (Ppib). Error bars display calculated minimum and maximum of SEM (SEM ± min, max) expression levels of four independent biological experiments, each measured in one or two technical replicates. *, $p < 0.05$; **$p < 0.01$; ***, $p < 0.001$ (vs. solvent control); $, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$ (vs. LPS treatment). Student's t-test was performed for statistical analysis.

Cox2 and iNos expression regulate production and release of respective signaling molecules, the effect of NE and GA on the release of NO, TxB_2 and different prostanoids have been measured (Fig. 3C–E). While basal NO (Fig. 3C), TxB_2 (Fig. 3D) and prostaglandin (Fig. 3E, white bars) levels remained unchanged in the presence of NE and GA, treatment with LPS significantly elevates these signaling molecules in the supernatant of RAW264.7 macrophages ($p < 0.0001$, Fig. 3C–E). The LPS-induced production of NO was significantly decreased from 32.2 ± 3.7 µM (LPS control) to 26.0 ± 5.8 µM ($p < 0.05$) by NE and even more effectively by GA to 6.2 ± 5.7 µM ($p < 0.01$; Fig. 3C, grey bars). The release of TxB_2 was inhibited in LPS-activated macrophages by NE and GA to 37.3% ± 25.2% ($p < 0.01$) and 9.1% ± 7.8% ($p < 0.001$) remaining activity, respectively (Fig. 3D, grey bars). In addition, the effect of NE and GA on the release of different prostaglandins was measured by ultraperformance liquid chromatography-coupled tandem mass spectrometry (UPLC-MS/MS). Co-incubation of LPS and GA decreased the release of PGE_2 and PGD_2 (Fig. 3E) almost to baseline levels, 0.09 ± 0.03 RU ($p < 0.05$) and 0.05 ± 0.02 RU ($p < 0.01$), respectively, whereas NE decrease the prostaglandin levels to 0.53 ± 0.10 RU (PGE_2) and 0.49 ± 0.11 RU (PGD_2). To exclude that the potent inhibition of Cox-derived eicosanoids in LPS-activated macrophages depends on a direct inhibition of Cox isoenzymes, we determined the effect of GA on the activity of isolated bovine Cox1 and human recombinant COX2 in a cell-free assay. GA did not affect Cox1 and only weakly inhibited Cox2 (89 ± 13% residual activity) at a concentration of 10 µM (data not shown).

3.4. Local anti-inflammatory effects of GA in atherosclerotic lesions of aortic sinus

Based on the significant anti-inflammatory effects of GA shown here *in vitro*, we investigated its impact on the development of atherosclerotic plaques in male $ApoE^{-/-}$ mice fed with high fat diet (HFD). Under our experimental conditions body and organ weight (Suppl. Fig. S4A) as well as the plasma lipid profile (Suppl. Fig. S4B) of mice remained unchanged. Focusing on plaque morphology and stability, no significant differences were detected for morphological parameters including total lesion size, necrotic core area and lipid content analyzed using Hematoxylin and Eosin (H&E) and Oil Red O (ORO) staining, respectively. Collagen content (PSR), vascular cell adhesion protein (VCAM)-1 and cluster of differentiation (CD) 68, a marker for macrophage infiltration, remained unchanged (Fig. 4A). Analysis of intraplaque inflammatory profile revealed no change in monocyte chemoattractant protein (MCP)-1 and interleukin (IL)1β levels. However, treatment with GA significantly decreased nitrotyrosine level - a marker for inflammatory stress - in atherosclerotic plaques to 50% compared to the control group (Fig. 4B and Suppl. Fig. S2, $p < 0.05$).

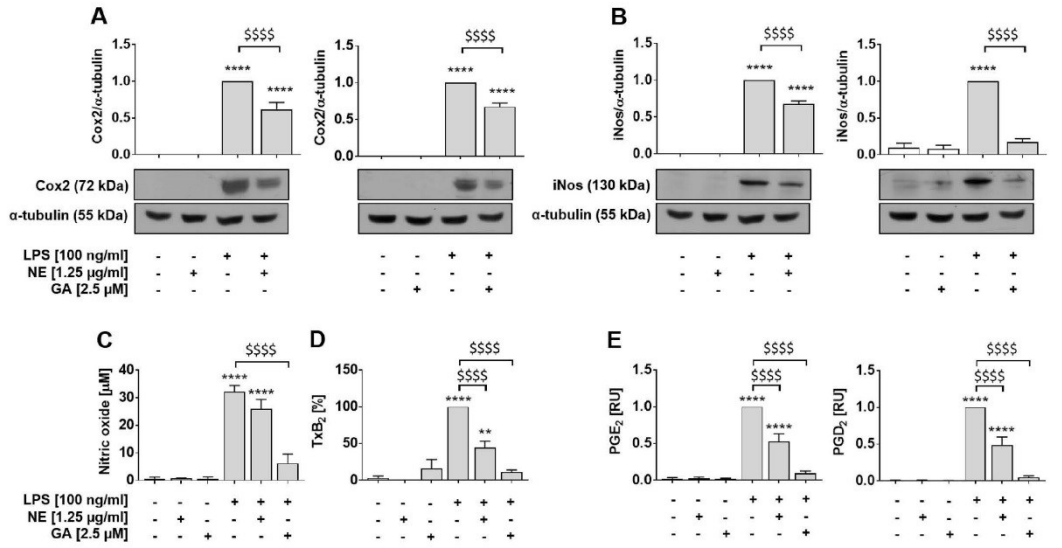

Fig. 3. Lipopolysaccharide-induced expression of iNos and Cox2 protein and secretion of respective signaling molecules are more potently blocked by GA compared to NE. RAW264.7 macrophages were incubated with either solvent (DMSO, white bars), NE or GA, or co-incubated with 100 ng/ml LPS (grey bars). The NE and GA decreased the protein expression of **(A)** Cox2 and **(B)** iNos after 24 h pre-incubation with NE or GA followed by 14 h and 24 h co-incubation with LPS, respectively. Samples incubated with LPS were defined as reference and were set as one. Protein levels were normalized to α-tubulin for quantification and representative Western blots are shown (for un-chopped versions see Suppl. Fig. S6). **(C)** Basal NO production, determined using Griess assay, were affected neither by the NE nor GA, whereas LPS-induced the formation of NO was significantly decreased by NE and even more effectively by purified GA. Treatment of RAW264.7 macrophages to measure released **(D)** TxB_2 and **(E)** PGs into culture supernatants followed the description in Fig. 2 except for use of 2.5 µM GA. Neither the NE nor GA altered the basal release of prostanoids. Treatment with LPS significantly induced TxB_2 and PG levels in the supernatant of macrophages and was set to 100% or one, respectively. Both, NE and GA decreased the release of TxB_2, PGE_2 and PGD_2 by LPS-activated macrophages Means of three independent biological experiments measured in two technical replicates **(A,B)**, three **(C)**, six **(D)** or four to five **(E)** independent biological experiments are shown; error bars display SEM. $^{*}, p < 0.05$, $^{**}, p < 0.01$, $^{***}, p < 0.001$ $^{****}, p < 0.0001$ (vs. control); $^{\$}, p < 0.05$, $^{\$\$}, p < 0.01$, $^{\$\$\$}, p < 0.001$, $^{\$\$\$\$}, p < 0.0001$ (vs. LPS treatment). Student's t-test and ANOVA followed by Tukey post-hoc tests with logarithmized values was performed for statistical analysis.

3.5. GA does not affect systemic levels of iNos and Cox signaling molecules

As shown in Fig. 3, GA significantly blocked Cox2 and iNos pathways in LPS-activated macrophages. To determine if these effects occur in our atherosclerotic mouse model, plasma levels of NO (Fig. 5A) and prostanoids (Fig. 5B–D) have been measured. Baseline prostanoid concentrations in plasma increased under atherosclerotic conditions 8-fold (TxB_2, $p > 0.01$), 1.4-fold (6 keto $PGF_{1\alpha}$, n.s.) and 8-fold (PGE_2, $p > 0.001$, Suppl. Fig. S5). However, treatment with GA did neither alter NO levels (Fig. 5A) nor changed prostanoid levels in mice (Fig. 5B–D).

3.6. GA differentially affects systemic and localized inflammation

Changes in the distribution of immune cells such as lymphocytes and monocytes and the modulation of their sub-populations play a pivotal role in atherosclerosis. Systemic (blood) and local (spleen) cell population have been changed after GA treatment in at least partly different extent. In blood, the total population of monocytes/macrophages as well as the pro-inflammatory $Ly6c^{high}$ and the anti-inflammatory $Ly6c^{low}$ monocyte sub-population remain unchanged (Fig. 6A). There is no change in B cell and CD8 T cell population. However, CD4 positive T cells are significantly downregulated in the GA-treated group by 14.8% ($p < 0.05$) and the CD4/CD8 ratio decreases from 2.1 ± 0.1 (control) to 1.9 ± 0.05 (GA treatment, $p = 0.077$). Further, natural killer (NK) and natural killer T (NKT) cells were significantly up-regulated 3.2% ± 0.4% vs. 5.6% ± 0.8%; 0.2% ± 0.02% vs. 0.6% ± 0.2%, respectively ($p < 0.05$, control vs. GA-treated group). In contrast, local (spleen) population of analyzed cell types remained unchanged, except for NK cells which were significantly upregulated (2.0% ± 0.1% vs. 2.8% ± 0.1%; $p < 0.001$, control vs. GA treatment).

4. Discussion and conclusions

Garcinia kola is known in Africa as a 'traditional' medicinal plant [7]. One of the significant phytochemicals found in *Garcinia kola* seeds is GA [7,18,19]. In order to obtain high yields of GA from *Garcinia kola* seeds, we developed an optimized extraction procedure for GA to ensure the most effective use of the seeds. Using our improved approach, we significantly increased the yield (6.6 fold) and purity (>99%) of extracted GA (Fig. 1 and Suppl. Fig. S1). Extracted GA is purer than the commercially available product as well as the GA received using former protocols, consequently indicating that the *Garcinia kola* seeds were utilized optimally by our extraction method. Having reasonable amounts of pure GA accessible opens new application possibilities, such as (i) *in vitro* and *in vivo* studies as well as (ii) using GA as a starting product for the synthesis of the long-chain metabolites of vitamin E (LCMs) as described by Mazzini et al. [6] Synthesis of the LCMs is essential to study their physiological role, as the LCMs are not commercially available.

The bioactivity of the *Garcinia kola* plant has been studied *in vitro* as well as in animal and human studies, as recently reviewed [22]. Different studies demonstrated the radical scavenging, anti-oxidative and

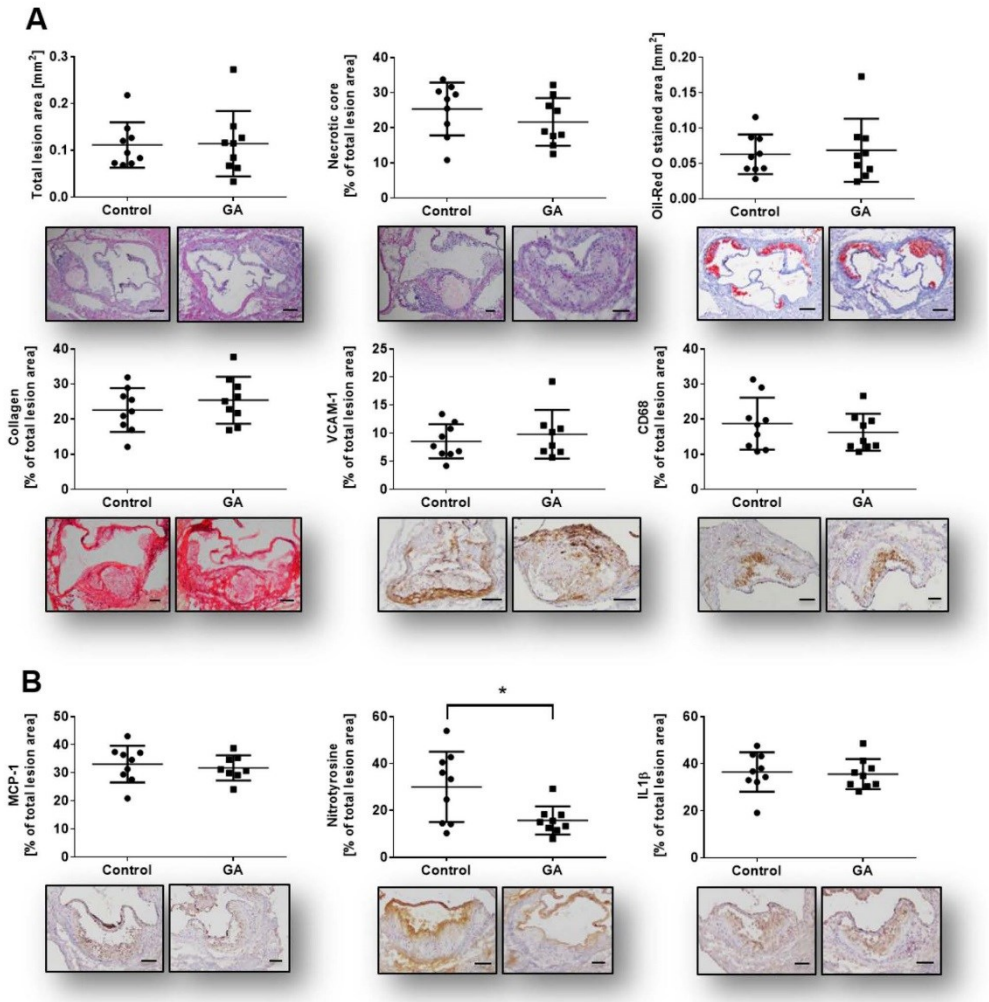

Fig. 4. Plaque morphology, stability and inflammatory profile of lesions. Frozen OCT embedded aortic sinus sections (6 µm) have been stained as follows: **(A)** Characterization of plaque morphology, stability and inflammation status has been analyzed using histological (Hematoxylin and Eosin; H&E, Oil Red O; ORO, Picro Sirus Red; PSR) and immunohistochemical staining (VCAM-1, CD68, MCP-1, nitrotyrosine, IL1β). No significant changes could be detected in all morphological parameters including total lesion size, necrotic core area (H&E), lipid content (ORO) and collagen content (PSR). GA application decreased inflammatory status as shown by a significant downregulation of nitrotyrosine level in the treatment group vs. control group. In contrast, adhesion marker (VCAM-1), macrophage content (CD68) and further inflammatory markers such as MCP-1 and IL1β remain unchanged. Single dots represent the mean of three to four sections per mouse. Error bars display calculated standard deviation. *, $p < 0.05$, scale bar 100 µm and 200 µm (total lesion size and ORO), magnification 100×. One-way ANOVA with multiple comparisons were calculated.

anti-inflammatory potential of the *Garcinia kola* plant, and the seeds in particular [3,7]. It has been shown that a methanol extract of the seeds reduces the LPS-induced NO production in the human macrophage cell line U937[3] and in rats [21], which is possibly mediated by one of its main phytochemicals, such as GA. Data on anti-inflammatory effects of isolated GA are rare. Until recently its anti-inflammatory potential was merely postulated based on its anti-oxidative effects [4]. To demonstrate the importance of GA for the reported anti-inflammatory effects of *Garcinia kola* seeds, we directly compared the effects of NE and GA *in vitro*. Our data revealed that both GA and NE interfere with anti-inflammatory signaling in LPS-stimulated RAW264.7 macrophages, with GA being slightly more efficient compared to NE (Figs. 2 and 3). Birringer et al. have shown that less than 1% of the *Garcinia kola* seeds is constituted by GA [18]. Since GA is soluble in organic solvents the percentage of GA in our NE is higher. Taking this into account, a significant contribution of GA to the anti-inflammatory effects of the NE is evident.

GA combines structural similarities of both, δ-T3 and the LCM of

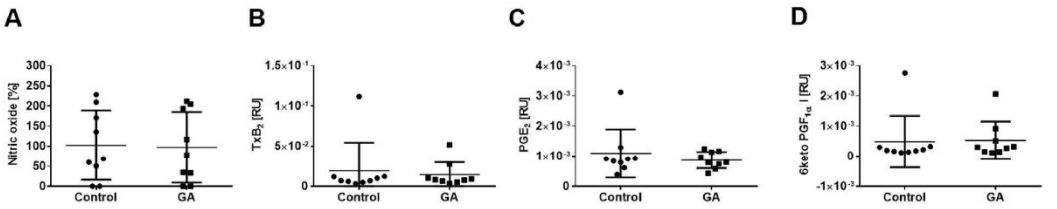

Fig. 5. GA does not affect systemic levels of iNos and Cox signaling molecules. Plasma samples from ApoE $^{-/-}$ mice fed with HFD and in parallel injected with GA or vehicle for eight weeks have been measured to determine **(A)** NO levels **(B)** thromboxane levels as well as **(C + D)** prostaglandin (PGE_2 and 6-keto $PGF_{1\alpha}$) levels. Mean of basal NO levels of vehicle control group was set as 100%. Treatment with GA did neither alter NO levels in mice (102.7% ± 28.8% vs. 97.1% ± 29.3%; control vs. treatment) nor the prostanoid levels (TxB_2: 1.939E-02 ± 1.162 E−02 vs. 1.443E-02 ± 0.523E-02, PGE_2: 1.092E-03 ± 0.2662 E−03 vs. 0.873E-03 ± 0.0908E-03, 6 keto $PGF_{1\alpha}$: 0.488E-03 ± 0.29 E−03 vs. 0.531E-03 ± 0.2088E-03; control vs. GA). Error bars display calculated SD. Student's t-test and ANOVA followed by Tukey post-hoc tests with logarithmized values was performed for statistical analysis.

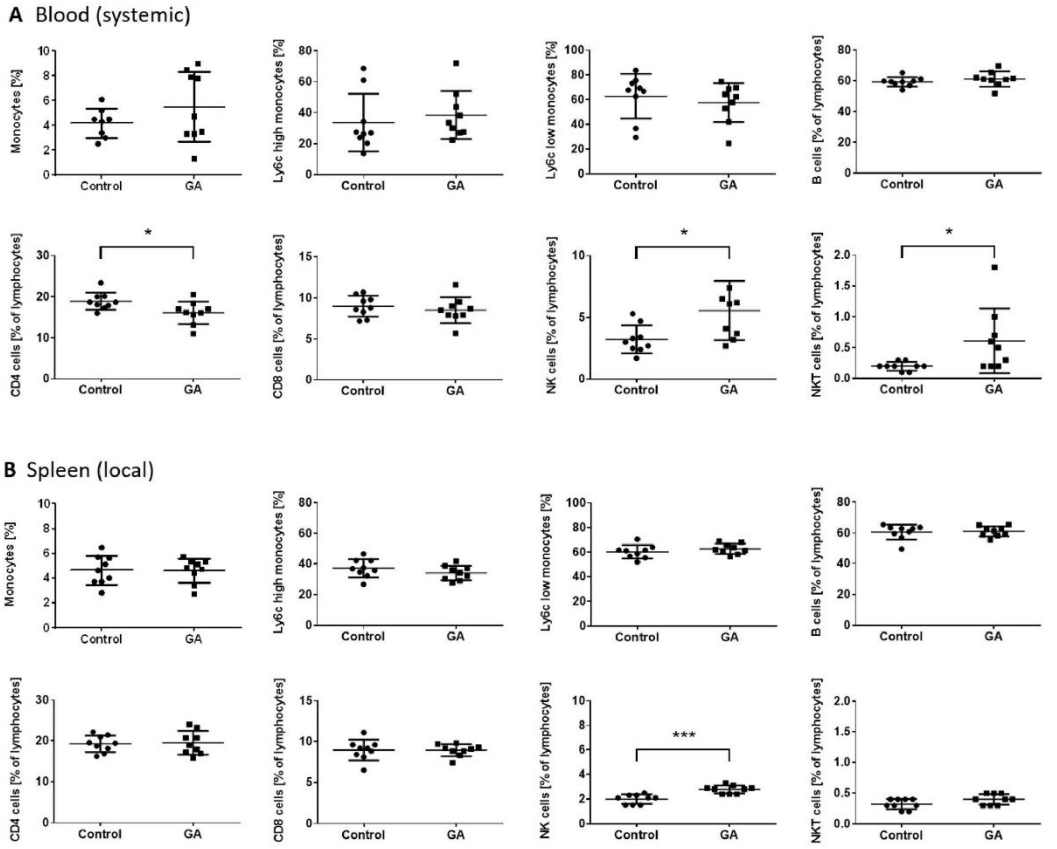

Fig. 6. GA modulates systemic and localized inflammation to different extent. Interactions of GA treatment with immune cell distribution were analyzed using flow cytometry. Systemic (blood) and local (spleen) cell population have been quantified using monocyte/macrophage, B cell and T cell specific fluorescent staining. **(A)** In blood total monocyte/macrophage population and sub-populations remained unchanged. There is no change in B cell and CD8 T cell population. CD4 positive T cells are significantly downregulated in GA-treated mice, 16.1% ± 0.9% compared to control group 18.9% ± 0.7%, whereas nature killer (NK) and natural killer T (NKT) cells were significantly upregulated 3.2% ± 0.4% vs. 5.6% ± 0.8%; 0.2% ± 0.02% vs. 0.6% ± 0.2%, respectively (control vs. GA-treated group). **(B)** In contrast local (spleen) population of tested cell types remained unchanged, except for an increase in NK cells (2.0% ± 0.1% vs. 2.8% ± 0.1%). Error bars display calculated SD. *, $p < 0.05$, ***, $p < 0.001$ (control vs. GA). Student's t-test was used.

vitamin E α-13′-COOH, with respect to the methylation pattern of the chromanol ring as well as the saturation and the terminal oxidation of the side chain, respectively. Within the group of vitamin E, δ-T3 is probably the most effective in inhibiting pro-inflammatory pathways [23]. For example, expression of Cox2, production of cytokines and release of PGE_2 and NO was inhibited in LPS-activated RAW264.7 macrophages by δ-T3 more effectively than by α-TOH, α-T3 and γ-T3 [9]. In line with these findings, Qureshi et al. have shown that Tnfα serum levels and expression of Il6, Il1β, iNos and Tnfα were effectively decreased by δ-T3 in LPS-stimulated peritoneal macrophages obtained from BALB/c mice [24]. Furthermore, GA (δ-TE-13′-COOH) has been described as the most potent 5-lipoxygenase (5-LO) inhibitor within the group of vitamin E metabolites [25].

α-13′-COOH is claimed to be a bioactive molecule since significant effects on lipid metabolism and homeostasis as well as inflammation have been reported [14,16,26,27]. In the study of Jiang and colleagues, the LCMs δ- and γ-13′-COOH were characterized as potent anti-inflammatory agents due to their ability to inhibit the COX2 pathway in IL1β-stimulated human lung adenocarcinoma A549 cells [14]. In addition, our group has recently shown that α-13′-COOH blocks the LPS-induced expression of inflammatory marker genes, proteins and related signaling molecules in LPS-stimulated RAW264.7 macrophages [16]. Similar anti-inflammatory properties have been recently discussed for the structurally related GA [28]. Here, we demonstrate that GA reduced the LPS-induced mRNA expression of iNos and other crucial inflammatory pathways (Fig. 2), followed by the inhibition of NO and other signaling molecules (Fig. 3C–E).

Based on the promising anti-inflammatory effects of GA *in vitro* and previous studies demonstrating anti-atherosclerotic effects of $T3s^{13}$, we tested the potential of GA as a treatment against the inflammation-driven disease atherosclerosis. For the *in vivo* application, a maximum dose of 1 mg/kg of GA was used; the dose of GA was chosen with respect to its low solubility in aqueous solutions and the need of using DMSO as solvent. We used the highest concentration of 0.8% DMSO that is allowed for long-term application in mice by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia. Due to the limited solubility of GA in aqueous solutions containing only 0.8% DMSO we had to limited the dose of GA to 1 mg/kg. Calculated to the total amount of blood (2 ml) in mice, with 1 mg/kg GA injected intraperitoneally, initial plasma concentrations of GA up to an equivalent of 35 μM could be achieved. We decided to use the highest possible dose of 1 mg/kg (35 μM) GA. However, we cannot exclude that the distribution and clearance of GA changes the actual concentration achievable *in vivo* and that higher concentrations are needed to achieve suitable concentrations *in vivo* resulting in anti-atherogenic properties of GA. We found a significant reduction of intra-plaque nitrotyrosine levels (Fig. 4B) – a marker for NO production as expected from our *in vitro* studies. Since elevated nitrotyrosine levels have been reported in human atherosclerotic lesions, its contribution to cardiovascular disease as a linking mechanism between inflammation and development of atherosclerosis has been discussed [29]. Other inflammatory marker, such as IL1β and MCP-1, remained unchanged in the plaque, although GA has been shown to block these pathways *in vitro* (Fig. 2C). The total plaque size was not affected by GA treatment, despite the apparent interference with local NO production. Overall, it can be assumed that the decrease of intra-plaque inflammation by GA was not sufficient to significantly impact the plaque size. As recently shown by Pein et al., GA potently inhibits 5-LO [25]. An association of the expression of 5-LO and the progression of atherosclerotic plaque formation [30], and more precisely plaque instability [31], in patients has been shown. However, there are discrepancies of 5-LO expression in atherosclerotic plaques of humans and mice. Whereas the expression of 5-LO is upregulated in carotid plaques in humans, there is no difference in the expression levels in wild-type mice (C57Bl/6) compared to atherosclerotic mice strains ($ApoE^{-/-}$ and $ApoE^{-/-}/LDLR^{-/-}$) fed a HFD [32]. Indeed, the $5\text{-}LO^{-/-}/ApoE^{-/-}$ knockout model or pharmacological inhibition of 5-LO in mice failed to show an impact on the progression of atherosclerosis [33]. Therefore, evidence for a contribution of 5-LO to the formation of atherosclerotic plaques is still on demand.

As shown in Fig. 3C–E, GA efficiently blocks the production of NO and prostanoids in LPS-activated macrophages *in vitro*. However, neither NO nor prostanoid levels are changed by GA in murine plasma samples. The atherosclerotic mouse model used in our study is characterized by a low-level chronic inflammation. Therefore, baseline NO and prostanoid plasma levels are at least 100-fold lower compared to levels in LPS-activated macrophages. The lack of excessive pre-activation could be a reason why we did not see inhibitory effects of GA on systemic pro-inflammatory signaling molecules in our study. Whether GA would be more effective in acute inflammation (e.g. sepsis), which is closer to our *in vitro* model, needs further investigation. We can also not exclude that a different treatment regime or animal model of atherosclerosis would lead to different results.

Monocytes play a pivotal role in atherogenesis. We found the total monocyte population and the $Ly6C^{low}$ and $Ly6C^{high}$ sup-populations in blood and spleen to be unchanged. Effects of α-TOH [34] and $\gamma\text{-}T3^{1,2}$ on monocyte and macrophage recruitment have been shown, respectively, but nothing was known for GA. In addition, we observed that lymphocyte populations, especially T cells, NKT and NK cells, are modulated under GA treatment. This demonstrates the importance of oxidative modifications of the side chain for vitamin E metabolites on regulatory effects. In blood, GA treatment decreased CD4 positive cells and the CD4/CD8 cell ratio without regulation the CD8 positive cell population. This finding goes in line with the earlier reported effects of vitamin E in Brown Norway rats [35] and chickens [36]. Further, NK and NKT cells, which are important for the defence against tumour cells, are significantly increased. Since these cells are known to regulate immune responses, their upregulation shows the involvement of GA on the immune system. In support, effects of α-TOH on increased NK cell activity and their tumorolytic activity in mice have been shown [37].

In summary, our improved procedure for the extraction of GA from *Garcinia kola* seeds enabled us to perform *in vitro* and *in vivo* investigations using pure GA. For the first time, we clearly demonstrate that both the NE and GA efficiently affect acute inflammation by inhibiting LPS-induced pro-inflammatory pathways *in vitro*. However, in atherosclerosis as a model of low level chronic inflammation this effect is not sufficient to make a significant difference in plaque size and plaque stability. Therefore, further studies are required to unravel the effects of GA in inflammation-driven diseases using different animal models to shed new light on the molecular modes of action of GA and to verify the *in vivo* importance of our findings.

Authors contribution

MW, JB, SK, LS, YCC, MZ, AS, AM, MS, MT, HP and AK performed the experiments. MW and SL designed the study. MW, MB, KP and SL supervised the project. MW wrote the manuscript. MW, JB, SK, LS, YCC, MZ, AS, AM, MS, AK, OW, MB, KP and SL carefully read and evaluated the manuscript and discussed the results.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101166.

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Research Paper

α-Tocopherol preserves cardiac function by reducing oxidative stress and inflammation in ischemia/reperfusion injury

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ABSTRACT

Objective: Myocardial infarction (MI) is a leading cause of mortality and morbidity worldwide and new treatment strategies are highly sought-after. Paradoxically, reperfusion of the ischemic myocardium, as achieved with early percutaneous intervention, results in substantial damage to the heart (ischemia/reperfusion injury) caused by cell death due to aggravated inflammatory and oxidative stress responses. Chronic therapy with vitamin E is not effective in reducing the cardiovascular event rate, presumably through failing to reduce atherosclerotic plaque instability. Notably, acute treatment with vitamin E in patients suffering a MI has not been systematically investigated.

Methods and results: We applied alpha-tocopherol (α-TOH), the strongest anti-oxidant form of vitamin E, in murine cardiac ischemia/reperfusion injury induced by ligation of the left anterior descending coronary artery for 60 min. α-TOH significantly reduced infarct size, restored cardiac function as measured by ejection fraction, fractional shortening, cardiac output, and stroke volume, and prevented pathological changes as assessed by state-of-the-art strain and strain-rate analysis. Cardioprotective mechanisms identified, include a decreased infiltration of neutrophils into cardiac tissue and a systemic anti-inflammatory shift from $Ly6C^{high}$ to $Ly6C^{low}$ monocytes. Furthermore, we found a reduction in myeloperoxidase expression and activity, as well as a decrease in reactive oxygen species and the lipid peroxidation markers phosphatidylcholine (PC) (16:0)-9-hydroxyoctadecadienoic acid (HODE) and PC(16:0)-13-HODE) within the infarcted tissue.

Conclusion: Overall, α-TOH inhibits ischemia/reperfusion injury-induced oxidative and inflammatory responses, and ultimately preserves cardiac function. Therefore, our study provides a strong incentive to test vitamin E as an acute therapy in patients suffering a MI.

1. Introduction

Myocardial infarction (MI) is the single most frequent cause of death worldwide [1]. Substantial progress in the treatment of MI has been achieved by early reperfusion strategies based either on pharmacological thrombolysis or percutaneous coronary intervention resulting in reperfusion of the ischemic myocardium. However, reperfusion itself causes additional damage to the myocardium, damage which has been estimated to contribute to about 50% of the overall functional loss of the infarcted heart [2]. This ischemia/reperfusion (I/R) injury is characterized by necrosis of myocardial tissue which is caused by a combination of extensive inflammatory and oxidative stress [3]. Infiltration of immune cells, particularly neutrophils [4] and monocytes [5], followed by the production and release of chemokines and cytokines, is a

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central component of I/R-induced inflammation.

One of the enzymes that contributes to oxidative stress in I/R injury is myeloperoxidase (MPO). MPO is stored in leukocyte granules, and is released by leukocyte activation during inflammatory reactions and oxidative stress [6]. MPO is elevated in patients with a MI compared to healthy subjects [7], and has therefore been discussed as a potential circulating biomarker for MI [8]. In addition, MPO causes endothelial dysfunction, and affects the function and distribution of cholesterol [9], lipid peroxidation, and oxidation of lipoproteins [10]. Oxidized lipids are excessively taken up by macrophages via non-feedback-regulated pathways, which in turn cause foam cell formation, apoptosis, and further release of these lipids. Therefore, accumulation of lipids and lipid peroxidation during ischemia and reperfusion is an initiator of lipotoxicity, which causes apoptotic cell death, cardiac dysfunction, remodeling, and ultimately heart failure [11].

One of the most effective anti-oxidant and anti-inflammatory agents is vitamin E and its derivatives. Vitamin E has eight derivative forms, which differ in the methylation of the chromanol ring and the saturation of the side chain. Within this group, α-Tocopherol (α-TOH) is known to be the most active anti-oxidant [12]. Besides protection against H_2O_2-induced lipid peroxidation due to increased anti-oxidative enzyme systems such as glutathione and catalase [13], α-TOH reduces oxidative stress-induced apoptosis [14]. In addition to its anti-oxidative capacities, α-TOH acts as a regulator of genes involved in lipid metabolism and homeostasis, inflammation [15] and the immune defense system, the latter demonstrated by boosting resistance against pneumococcal infection [16]. Indeed, infection-induced transepithelial migration of neutrophils in the lung is reduced by α-TOH [17]. Furthermore, α-TOH prevents macrophage foam cell formation [18,19], lipotoxicity in macrophages [20], and the release of pro-inflammatory cytokines [21].

A recent study of Huang et al. reported that a higher α-TOH serum concentration correlates with decreased all-cause mortality and disease-specific mortality, such as cardiovascular disease and heart disease [22]. As the plasma levels of vitamin E decrease in patients within the first 48 h after MI [23–25], and as I/R injury is associated with excessive oxidative stress, increased consumption of this anti-oxidant in the ischemic and reperfused myocardium has been postulated [26,27]. Supplementation of vitamin E as a strong anti-oxidant may thus represent a therapeutic option for anti-oxidative protection of the myocardium and ultimately for patients suffering a MI. Vitamin E supplementation did not fulfil its original promise in several large-scale trials aimed at assessing its potential in primary and secondary prevention of cardiovascular events [28]. However, mechanistically these trials tested for vitamin E's capacity to provide plaque stabilization in a chronic setting, but not its potential to preserve cardiac function in the event of an acute MI. There is only very limited data available that addresses the question of the potential benefits of vitamin E in the acute setting of a MI [29,30]. Nevertheless, the limited data available shows potential benefits of vitamin E in models of ischemia/reperfusion settings of various organs, including a few early studies on cardiac ischemia/reperfusion.

In our study, we systematically address this clinically important question in a mouse model of cardiac I/R using a 60 min ligation of the left anterior descending (LAD) coronary artery. Using extensive echocardiographic and histological assessment to determine cardiac function and injury, in addition to thorough molecular and mechanistic studies, we demonstrate a cardioprotective effect of vitamin E supplementation in cardiac ischemia/reperfusion injury.

2. Materials and methods

2.1. Animals

C57BL/6 mice were acquired from Jackson Laboratories and bred by the Alfred Medical Research and Education Precinct (AMREP) Animal Services in Melbourne, VIC. All experimental work was performed in accordance with the *guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes* and the *Australian code for the care and use of animals for scientific purposes* and was approved by the AMREP Animal Ethics Committee (E/1779/2018/B).

2.2. Myocardial ischemia/reperfusion injury in mice

Eight-week-old male C57BL/6 mice underwent open-chest surgery to induce left coronary artery occlusion (CAO) for 60 min, followed by reperfusion as previously described [31]. Beforehand, mice were anesthetized using a combination of ketamine HCl (100 mg/kg BW; Lyppard), xylazine HCl (5 mg/kg BW; Lyppard), and atropine (1 mg/kg BW; Pfizer) via a single intraperitoneal (IP) injection. Randomized mice were intraperitoneally (IP) injected with either a vehicle (PBS with 0.8% DMSO) or α-TOH (2.5 mg/kg BW in 0.8% DMSO; Sigma-Aldrich) 2 h prior to surgery, immediately after reperfusion, and twice per day for three consecutive days. Following surgery, mice were culled at three different time points for respective analysis, using a ketamine HCl (100 mg/kg BW; Lyppard)/xylazine HCl (20 mg/kg BW; Lyppard) overdose IP injection followed by cervical dislocation. More details are given in the online-only Data Supplement.

2.3. Histology and immunofluorescence

Hearts were harvested, fresh-frozen in OCT Tissue Tec (Sakura® Finetek), and cut into 6 μm sections (Microm HM 525 Cryostat, Thermo Fisher Scientific). Cardiac sections were stained to detect neutral lipid content using Oil Red O (ORO, Sigma-Aldrich). To analyze neutrophils, tissue sections were stained using rat anti-mouse Ly6G (Gr-1) monoclonal antibody (ebioscience), followed by secondary Alexa Fluor 546–labeled anti-rat antibody (Life Technologies), and Hoechst 33342 dye counterstaining (Thermo Fisher Scientific). More details are given in the online-only Data Supplement.

2.4. Flow cytometry

Antibodies were purchased from BD Bioscience if not otherwise indicated. Blood samples were taken in 0.5 M anti-coagulant ethylenediaminetetraacetic acid (EDTA) by cardiac puncture. Blood was centrifuged (300 × *g*, 10 min, RT) to separate the plasma. Within 1 h of collection, cells from the blood were isolated and stained for flow cytometric analysis. Neutrophil and monocyte populations in the blood were analyzed using a FACS Canto II (BD Biosciences) and BD FACS DIVA software version 8.0.1. The total monocyte/macrophage population was detected using fluorescent anti-CD11b-FITC and anti-CD115-PE-Cy7 antibodies (Biolegend). For separating pro- and anti-inflammatory monocyte sub-populations, Ly6C-PB staining was performed in parallel. Neutrophils were gated using Ly6G (Gr-1)-PE staining.

2.5. Ribonucleic acid (RNA) isolation and PCR arrays for inflammatory cytokines and oxidative stress

The apex of each heart was collected, and three samples were pooled and used for RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and converted to cDNA using the RT^2 First Strand Kit (Qiagen) according to the manufacturer's instructions. Inflammatory cytokine and oxidative stress gene expression profiles were analyzed using the 384 well RT^2 Profiler™ PCR Array Mouse Inflammatory Cytokines & Receptors and the RT^2 Profiler™ PCR Array Mouse Oxidative Stress and Antioxidant Defense (Qiagen), respectively. The arrays were performed using QuantStudio 6k Flex (Applied Biosystems), and the GeneGlobe Data Analysis Centre (Qiagen) was used for data analysis.

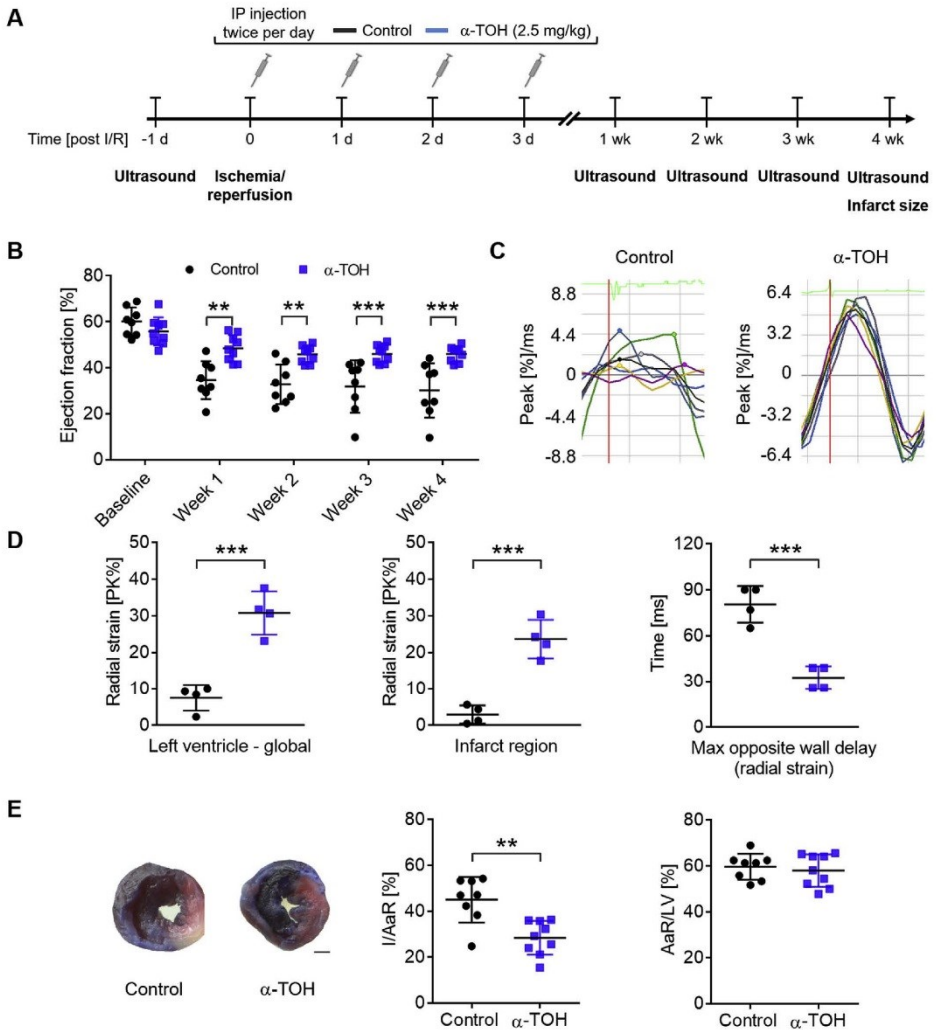

Fig. 1. Treatment with α-TOH protects cardiac systolic function and reduces infarct size in a mouse model of I/R injury. A) Design of long-term study (28 days post-I/R injury). **B)** α-TOH treatment preserves cardiac function assessed by ejection fraction from week 1 to week 4 after I/R injury; n = 8–9, $^{**}p < 0.01$ and $^{***}p < 0.001$, one-way Anova with multiple comparison. **C)** Representative images of radial strain curves obtained from VevoStrain analysis software shows strain measures over time. Colored lines represent 6 standard myocardial regions; 7th black line calculates average (global) strain at each time point. **D)** Bar charts show significant decrease in radial strain for control animals, as compared to α-TOH-treated animals, both globally and in infarct area (anterior apex). Maximum opposite-wall delay shows significant increases in time for control animals, as compared to α-TOH-treated animals; n = 4, $^{***}p < 0.001$, Student's *t*-Test. **E)** Representative images of Evans blue/TTC staining 28 days post-I/R injury (scale bar: 1 mm) and quantitative analysis of infarct size (I) per area at risk (AaR), which illustrates a significant decrease in infarct size in mice treated with α-TOH as compared to control animals, while the primarily affected area presented in % AaR/LV is similar between α-TOH treated mice and control mice. Data are presented as means ± SD, $^{**}p < 0.01$ and $^{***}p < 0.001$, n = 8–9, Student's *t*-Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. Lipid measurement

Blood was collected in EDTA as described above. Total serum cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides (TG) were measured with commercial enzymatic kits using a COBAS Integra 400 Plus blood chemistry analyzer (Roche Diagnostics). The instrument was calibrated on the day of use according to the manufacturer's instructions.

2.7. Lipid extraction

Prior to lipid extraction, samples were randomized and blinded.

Lipids were isolated from the infarct area of the cardiac tissue samples (36–50 mg) using a single-phase chloroform/methanol extraction as previously described [32]. Lipid analysis was performed by liquid chromatography electrospray ionization-tandem mass spectrometry using an Agilent 1290 HPLC coupled to an Agilent 6490 triple-quadrupole mass spectrometer. Lipid extracts were injected and separated under gradient conditions. The oxidized lipid species phosphatidylcholine (PC) (16:0)-9-hydroxyoctadecadienoic acid (HODE)) and PC(16:0–13-HODE) were measured using dynamic multiple-reaction monitoring (dMRM) and analyzed using Mass Hunter Quantitative analysis version B.07. Relative lipid abundances were calculated by relating each area under the peak for each lipid species (Avanti Polar Lipids, Alabaster, US) to the corresponding internal standard. Correction factors were applied to adjust for different response factors, where these were known. Results are expressed as pmol/mg of heart tissue. Values for each lipid class were calculated as the sum of the individual lipid species. More details are given in the online-only Data Supplement.

2.8. MPO activity assay

The infarct area of cardiac tissue samples (36–50 mg) were collected in ice-cold MPO assay buffer (50 µl/10 mg). After mechanical disruption, cardiac tissues were homogenized three times for 30 s at 30/s using a TissueLyser II (Qiagen). Immediately after homogenization, undiluted fresh samples were used in duplicate for MPO activity measurement using an MPO Fluorometric Activity Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Released fluorescein was measured every 15 min and respective MPO activity was calculated using a fluorescein standard curve.

2.9. In vivo reactive oxygen species (ROS) quantification using a fluorescent nanoprobe

Three mice per treatment group underwent I/R injury by CAO for 60 min followed by reperfusion for another 24 h. Recently described [33] ROS-sensitive nanoparticles (5 µg/g BW) were intravenously injected 20 min before mice were euthanized and perfused with PBS; the hearts and blood were then collected. Each heart was cut into four transverse sections and imaged from both sides using an IVIS Lumina XRMS system (PerkinElmer). The 2D scans were performed using the following settings: filter passband = excitation at 420 nm and emission at 670 nm. Results are expressed as total radiance [p/s]/[µW/cm^2] levels per g of tissue/blood. Cardiac sections were stained with 1% triphenyltetrazolium chloride (TTC) for 10 min at 37 °C in darkness and scanned using a high-resolution scanner (Epson Perfection Photo Scanner V370) to detect the infarct areas.

2.10. Statistical analysis

Data were statistically analyzed using one- or two-way repeated-measures ANOVA. As a *post hoc* test, Bonferroni's multiple comparisons test was used. P values of less than 0.05 were considered statistically significant. Results are expressed as means ± standard deviations (SD).

3. Results

3.1. Protection of cardiac function and reduction of infarct size after α-TOH treatment

Mice were subjected to cardiac I/R injury to assess the cardioprotective effects of α-TOH as a potential treatment for MI (Fig. 1A). Application of 2.5 mg α-TOH/kg BW twice per day for three consecutive days significantly increased systemic concentration of α-TOH (Supplemental Fig. S1). Weekly echocardiography was performed to assess changes in cardiac function. For precise echocardiographic measurements of left ventricular (LV) function, we used both parasternal long-axis and parasternal short-axis views. At baseline, conventional echocardiographic measures showed similar ejection fractions (EF) for both treatment groups (control: 60.1 ± 6.2 versus [vs] α-TOH: 55.9 ± 6.1% EF, mean ± SD, NS; Fig. 1B). The cardioprotective effect of α-TOH treatment compared to controls was already significant at week 1 post-I/R injury (34.6 ± 8.3 vs 48.5 ± 5.7; **p < 0.01). Similar results were obtained at week 2 (32.8 ± 8.6 vs 45.9 ± 3.7; **p < 0.01), week 3 (31.9 ± 11.4 vs 46.1 ± 3.8; ***p < 0.001), and week 4 (30.2 ± 11.8 vs 46.1 ± 3.3; ***p < 0.001). The measurements of fractional shortening, cardiac output, and stroke volume also showed significant cardioprotective effects of α-TOH (Supplemental Fig. S2A).

At week 4, compared to baseline, cardiac output (***p < 0.001) and stroke volume (*p < 0.05) showed significant decreases in the PBS control group as compared to the α-TOH-treated animals (Supplemental Figs. S2B and 1C). There was no significant difference between the two groups for heart rate at baseline or at week 4 post-I/R surgery (Supplemental Fig. S2D). Further central echocardiographic measurements showed a decrease in LV internal diameter at end diastole (p = 0.05) and end systole (*p < 0.05) in the α-TOH group compared to the control at week 4 (Supplemental Fig. S3). No difference was observed for both the LV interventricular septal wall and the LV posterior wall.

For a more sensitive and a highly translationally relevant readout, we decided to perform strain and strain-rate analyses [34]. We observed deterioration of the strain pattern in the control mice, while α-TOH treated mice preserved a physiological strain pattern. Radial strain analysis showed a highly significant decrease in control mice as compared to α-TOH-treated mice, both globally (control: 7.5 ± 3.5 vs α-TOH: 30.8 ± 5.9% PK, ***p < 0.001) and in infarct areas (anterior and apex) (control: 3.0 ± 2.5 vs α-TOH: 23.7 ± 5.3% PK, ***p < 0.001). Maximum opposite-wall delay showed significant increases in time for the control group as compared to the α-TOH group (control: 80.5 ± 12.0 vs α-TOH: 32.5 ± 7.5 ms, ***p < 0.001; Fig. 1C and 1D). Longitudinal strain analysis of the global peak (***p < 0.001) and the infarct area (*p < 0.05) obtained similar differences as in the radial strain analysis (Supplemental Fig. S4).

Histological evaluation of infarct size using Evans blue/TTC staining at week 4 post-I/R injury is a valuable parameter for evaluating the efficacy of interventions and cardiac performance. α-TOH-treated mice showed a significant decrease in infarct size (I)/area at risk (AaR) ratio as compared to controls (28.4 ± 7.4 vs 45.0 ± 9.9% I/AaR, respectively; **p < 0.01), while the AaR showed a similar size in all treatment groups, indicating comparable surgical procedures with similar sites of the LAD being ligated (AaR/LV; Fig. 1E).

3.2. Changes in blood cell profile and mRNA expression of inflammatory cytokines and receptors

A central part of cardiac I/R injury is a massive pro-inflammatory response in the infarcted myocardium, including inflammatory cell infiltration, cytokine production, and oxidative stress. To study the underlying mechanism systemically and locally, we performed LAD ligation surgeries and investigated the above listed processes in the blood and myocardium at day 1 and 3 post-I/R injury (Fig. 2A).

Changes in monocyte and neutrophil counts in the blood due to treatment with α-TOH were assessed by flow cytometric analysis after I/R injury (Fig. 2B and C and Supplemental Fig. S5). Compared to control mice, mice treated with α-TOH showed approximately a 50% reduction in blood monocytes at day 1 post-I/R. Importantly, subtype analysis determined a significant shift from pro-inflammatory Ly6C^{high} monocytes toward anti-inflammatory Ly6C^{low} monocytes at day 1 and day 3 post-I/R. Furthermore, neutrophils were significantly decreased in the blood of α-TOH-treated mice at both time points.

At day 3 post-I/R injury, gene expression profiling revealed that α-

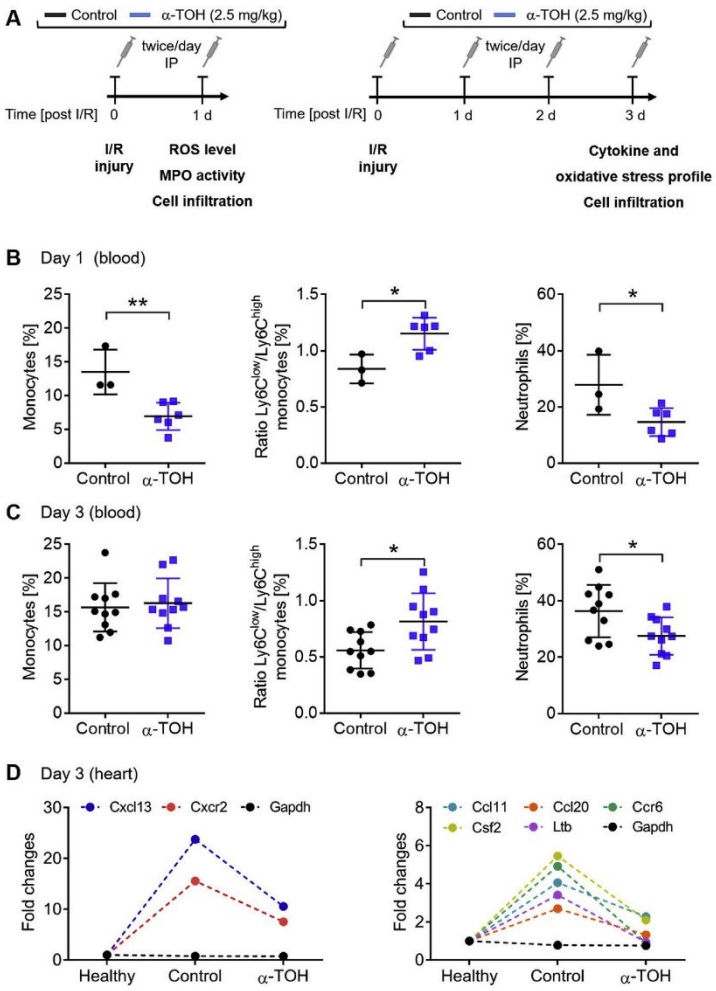

Fig. 2. Decrease in systemic and local inflammation in α-TOH-treated animals. A) Design of short-term studies (1 and 3 days post-I/R injury). **B)** α-TOH treatment reduces systemic inflammation, as assessed in the blood, by reducing total monocyte and neutrophil counts, and shifting the monocyte ratio from pro-inflammatory $Ly6C^{high}$ monocytes toward anti-inflammatory $Ly6C^{low}$ monocytes at day 1 after I/R injury; n = 3–6, *p < 0.05 and **p < 0.01, Student's *t*-Test. **C)** At day 3 post-I/R, the total monocyte population in the blood is unchanged, although the ratio is still shifted toward anti-inflammatory $Ly6C^{low}$ monocytes. A significant reduction in neutrophils was also observed; n = 10, *p < 0.05, Student's *t*-Test. **D)** Local cytokine responses in the myocardium for α-TOH-treated mice, control mice, and healthy mice. Expressions of 7 of the 84 tested genes are strongly upregulated after I/R and diminished in the α-TOH-treated animals compared to the control group; n = 10.

TOH treatment, compared to the controls, diminished the expression of seven inflammatory cytokines and receptors, which were significantly upregulated after control-treated I/R injury (Fig. 2D). For example, Cxcr2, a receptor expressed on neutrophils that mediates neutrophil migration to sites of injury and inflammation, as well as CCl11, a neutrophil chemoattractant, were strongly downregulated. This observation is in line with the reduction of neutrophils in the blood, as well as the reduction of neutrophils in the infarcted myocardium (see below).

α-TOH reduces oxidation of lipids in the myocardium independent of neutral lipid accumulation and systemic lipid profile.

In addition to the inflammatory areas, we investigated the plasma lipid profile and changes in oxidized lipids as markers for oxidative stress in the infarcted myocardium. As well as systemic changes, the heart undergoes structural and functional changes in response to I/R injury, which lead, for example, to changes in the neutral lipid profile as well as the reduction of oxidation of several lipids.

First, we measured plasma lipids at day 1 and day 3 post-I/R and observed no changes in TC, TG, HDL, and LDL between treatment groups (Fig. 3A). Total lipid content in the heart was determined using ORO staining followed by quantitative analysis and showed no difference in total lipid content in the myocardium at three days post-I/R (Fig. 3B). Two major oxidized lipid species, namely PC(16:0–13-HODE) and PC(16:0-9-HODE), were assessed using liquid chromatography electrospray ionization-tandem mass spectrometry. Both oxidized lipids were significantly increased in the infarcted myocardium at day 3 post-I/R in comparison to healthy control samples (13-HODE: 32.3 ± 2.2 vs 44.3 ± 6.8 and 9-HODE 24.5 ± 2.0 vs 32.3 ± 4.4 pmol/mg heart tissue, respectively; ****p < 0.0001). A significant decrease in oxidized PC (16:0–13-HODE) (control: 44.3 ± 6.8 vs α-TOH: 37.7 ± 3.0 pmol/mg heart tissue; **p < 0.01) and a trend toward reduction of oxidized PC (16:0-9-HODE) (32.3 ± 4.4 vs 28.9 ± 3.4 pmol/mg heart tissue) were found in the hearts of α-TOH-treated mice compared to control animals (Fig. 3C).

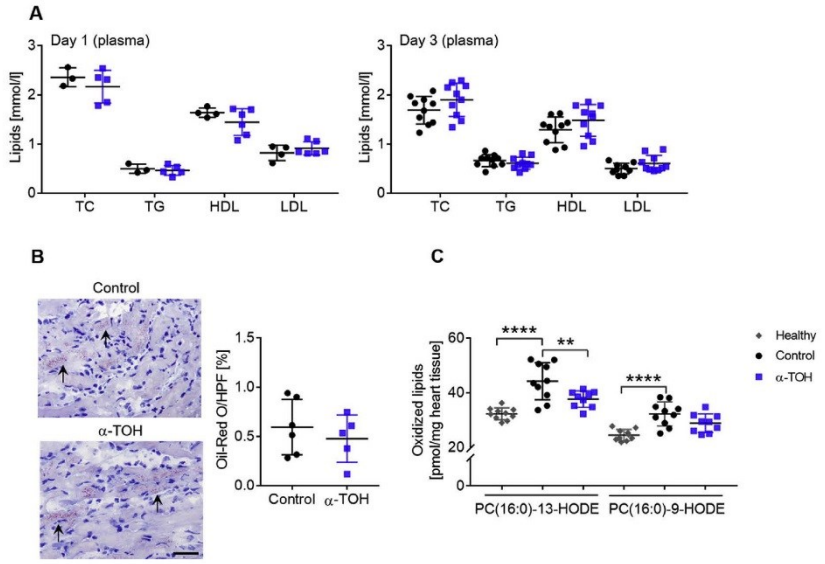

Fig. 3. Changes in systemic and local lipids, and oxidized lipids in mice in response to α-TOH treatment. A) Plasma lipids measured 1 and 3 days post-I/R are unchanged in α-TOH-treated mice compared to control mice; n = 4–10, one-way Anova with multiple comparison. **B)** Representative images and quantitative analysis show comparable lipid content in the myocardium as assessed by ORO staining. Scale bar: 30 μm, 200x magnification, n = 5–6, Student's *t*-Test. **C)** Oxidized lipids in the infarcted area of the myocardium are strongly increased three days after I/R injury and this effect is partly inhibited in mice treated with α-TOH as compared to control mice; n = 9–10, $^{**}p < 0.01$ and $^{****}p < 0.0001$, one-way Anova with multiple comparison.

3.3. Decrease in cardiac ROS production in α-TOH-treated mice

Both ischemic events as well as reperfusion injury lead to excessive ROS production, which ultimately causes tissue damage. With a reduction in oxidized lipids, here we have provided the first evidence that α-TOH treatment leads to an anti-oxidative response in the myocardium after I/R injury. To study this in more detail, we injected a ROS-sensitive nanoprobe, with its fluorescence quenched under normal conditions, while it was able to accumulate in the I/R injury area and ROS-activated fluorescence was then detected using IVIS imaging. Most importantly, IVIS showed a significant decrease of the ROS-dependent fluorescent signal in the α-TOH-treated ischemic/reperfused myocardium compared to control tissue ($5.1 \times 10^{11} \pm 0.5 \times 10^{11}$ vs $3.5 \times 10^{11} \pm 0.8 \times 10^{11}$ radiance level/g heart tissue; $^{**}p < 0.01$). No relevant radiance level was detected for whole blood in all groups (Fig. 4A). The cardiac section was stained with TTC to demonstrate the co-localization of the ROS signal with the infarcted area (Fig. 4B).

Regulation of oxidative stress-related genes, reduction in MPO activity, and decrease in neutrophil infiltration after α-TOH treatment.

To further confirm the reduction in ROS within the ischemic/reperfused myocardium by α-TOH treatment, the expression of genes involved in oxidative stress were profiled and it was found that the expression of seven oxidative stress-regulating genes were strongly downregulated in α-TOH-treated mice at day 3 post-I/R injury (Fig. 5A). Notably, several glutathione peroxidases (Gpx1, 5, and 6) were downregulated and, most strikingly, MPO was downregulated by more than 43% in mice treated with α-TOH as compared to control mice. Therefore, we continued studying the changes in MPO due to the strong downregulation of its expression, and performed MPO activity measurements in the I/R myocardium at day 1 post-I/R injury. In line with the downregulation of MPO expression, we found a significant decrease in MPO activity of about 40% in the α-TOH-treated group as compared to the control group (0.84 ± 0.3 vs 1.4 ± 0.3 μU/ml, respectively; $^{*}p < 0.05$; Fig. 5B). As MPO is well-known to be highly correlated with neutrophils, the number of infiltrating neutrophils within the infarcted area was determined and the infiltration of neutrophils was found to be strongly reduced in the infarcted tissue of mice treated with α-TOH (6.28 ± 1.9 vs 11.6 ± 3.9% neutrophils/infarct area, respectively; $^{**}p < 0.01$; Fig. 5C).

In summary, using a murine I/R injury model we demonstrate the potential of α-TOH in preventing tissue damage and retaining cardiac function after MI. α-TOH protects the heart against **(i)** oxidative stress-induced tissue damage such as decreased oxidative lipids, reduction in ROS production, and downregulation of the expression of oxidative stress-related genes. Furthermore, it induced **(ii)** anti-inflammatory changes, such as shifting the ratio of monocyte subpopulations toward anti-inflammatory $Ly6C^{low}$ monocytes and decreasing the number of infiltrating neutrophils (see graphical abstract).

4. Discussion

The excessive oxidative stress during MI [23,24,26], particularly during reperfusion [27] is associated with a drop in the anti-oxidant defense levels as reported for hydrophilic (vitamin C [35]) and lipophilic vitamins (α-TOH [25,26,36]) circulating in the blood. To compensate for this drop systemically and in the myocardial tissue [37,38], and also to provide the maximal anti-oxidative effect for the cardiac tissue undergoing ischemia and following reperfusion, we applied α-TOH as a treatment during the MI and then for three consecutive days. Our treatment regime reflects clinical conditions, where MI patients could receive their first application of α-TOH either in the ambulance or upon their arrival in the emergency department, before they are transported to the catheter laboratory for reperfusion by percutaneous coronary intervention, and the following days in hospital before discharge.

I/R injury-induced cardiac tissue damage is mainly caused by

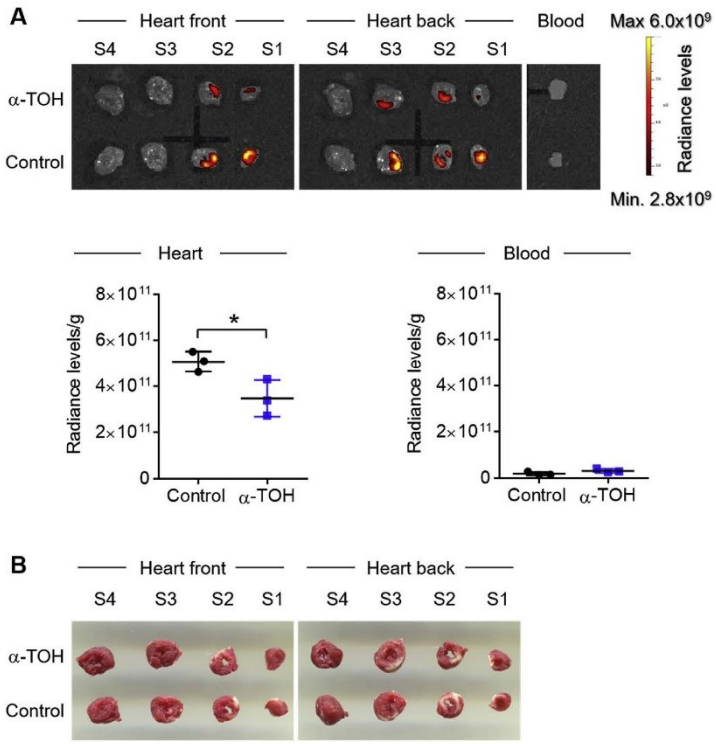

Fig. 4. Treatment of α-TOH reduces production of ROS after I/R injury. A) 2D IVIS scan and quantitative analysis of cardiac sections and blood, 1 day post-I/R. Fluorescence accumulation of a ROS-sensitive fluorescent nanoparticle is significantly reduced in I/R injury area of α-TOH-treated mice compared to control mice (scale bar: 5 mm). Whole blood samples display no accumulation of the dye in all groups; n = 3, *$p < 0.05$, Student's *t*-Test. **B)** Representative scans of TTC-stained cardiac sections show infarcted areas within the cardiac sections (scale bar: 5 mm).

significant leukocyte infiltration, particularly of monocytes [39] and neutrophils [40], and the subsequent release of pro-inflammatory chemokines and cytokines, as well as ROS. Therefore, inhibiting leukocyte migration is an attractive strategy in finding novel therapeutics for MI. α-TOH has been described as having immunomodulatory ability by affecting monocyte and neutrophil migration into inflamed areas in the lung [17]. In accordance with this, our study shows that α-TOH treatment shifts the monocyte profile in favor of anti-inflammatory $Ly6C^{low}$ monocytes systemically, and lower neutrophil infiltration locally in the ischemic myocardium, leading to a reduced infarct size and preserved cardiac function. As reported earlier, neutrophil migration and its lipoxygenase-dependent cytokine production enhance the inflammatory burst and thereby increase the expression of pro-inflammatory genes. In parallel, the interaction of neutrophils with ROS during I/R injury [41] has shown the multifactorial importance of neutrophils and neutrophil-derived signaling molecules as further promising targets for MI therapy.

Increased cardiac ROS formation and related signaling-pathway activation during I/R injury are a result of excessive oxidative stress [42–44]. Mitochondria play a pivotal role in ROS formation and mitochondrial membrane potential is an adequate marker for their metabolic activity. As described by Birringer et al., 10 μM α-TOH regenerated mitochondrial membrane potential, followed by decreasing cellular ROS formation in liver HepG2 cells [45]. In addition, formation of superoxide anion has been shown to be inversely correlated with α-TOH content in mitochondria *in vitro* and *in vivo* [46]. Protective effects of α-TOH on mitochondrial integrity preserved heart function and improved recovery following I/R [47]. Cardiac tissue is vulnerable to oxidative stress due to the low rates of expression of hydrophilic anti-oxidative detoxification systems [35,48]. However, lipophilic anti-oxidants such as vitamin E are normally abundant in cardiac tissue [47]. Induction of anti-oxidant defense mechanisms after I/R, such as increases in superoxide dismutase, glutathione transferase, and catalase, can be induced by α-TOH [49,50] whereas α-TOH deficiency in heart tissue enhances ROS formation [48,51]. We have studied ROS formation using an innovative ROS-sensitive nanoparticle [33]. Visualization of this ROS-sensitive fluorescent nanoprobe using *in vivo* imaging shows that I/R-induced ROS formation is decreased by α-TOH, confirming the ROS-scavenging capacity of α-TOH. In support of this hypothesis, as we have shown, α-TOH downregulated the ROS-induced gene expression of MPO in myocardial tissue, specifically its release from neutrophil granules [6]. Furthermore, the partial inhibition of neutrophil migration in the myocardial tissue by α-TOH contributes to the reduced release of MPO.

Excessive ROS production followed by MPO release also causes oxidative modification of cellular macronutrients such as lipids. Malondialdehyde [52], thiobarbituric acid reactive species [49], and hydroperoxides [47,52] are common markers for ROS-induced lipid peroxidation. Enhanced lipid peroxidation after I/R injury and the ability of anti-oxidants to attenuate oxidative stress-induced lipid

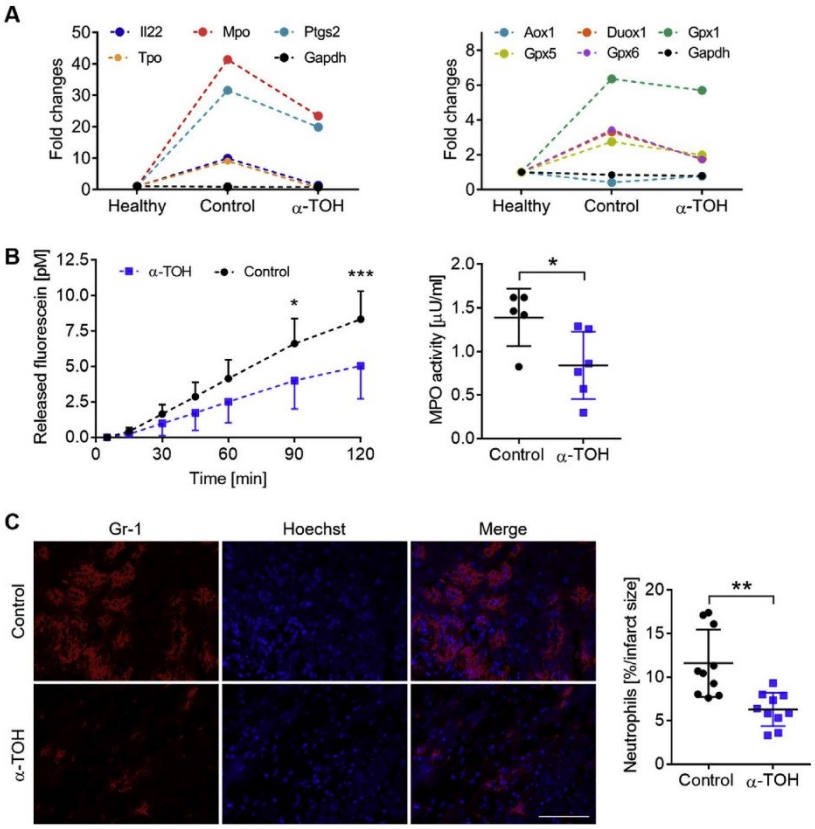

Fig. 5. Modification of expression of oxidative stress-regulated genes, MPO activity, and neutrophil infiltration. A) Oxidative stress profiler array analysis with cardiac tissue samples for α-TOH-treated mice, control mice, and healthy mice. Seven oxidative stress-related genes show strong upregulation after I/R injury; this upregulation is diminished by α-TOH treatment; n = 10. **B)** Time course of released fluorescein representing MPO activity and quantitative analysis of average MPO activity, which show a decrease in MPO activity in α-TOH-treated mice compared to control mice; n = 6, *$p < 0.05$ and ***$p < 0.001$, one-way Anova with multiple comparison and Student's *t*-Test. **C)** Representative images and quantitative analysis display a reduction of infiltrating neutrophils in the myocardium. Cardiac sections are counterstained with Hoechst dye. Scale bar: 100 µm, 200x magnification, n = 10, **$p < 0.01$, Student's *t*-Test.

peroxidation have been reported previously [49,53]. Vitamin E-deficient rats are characterized by significantly higher levels of myocardial lipid peroxidation [54], which is antagonized by α-TOH treatment. One of the major membrane-associated lipid classes prone to be oxidized by ROS is PC, due to its high content of polyunsaturated fatty acyl chains [55]. In addition, oxidized *PC*-containing phospholipids, generated in cardiomyocytes during I/R, affect the viability of cardiomyocytes and therefore increase infarct size [56]. Treatment with α-TOH has been shown to modulate PC metabolism via phospholipase A [57]. Here, we report and apply a novel technique to measure lipid products directly oxidized during I/R injury using liquid chromatography electrospray ionization-tandem mass spectrometry. Lipid profiles in plasma and the accumulation of lipids, particularly triglycerides, in ischemic myocardial tissue remained unchanged in our experimental setup, however, α-TOH treatment significantly decreased the oxidative modification of lipids. Specifically, α-TOH decreased I/R injury-induced formation of oxidized PC, namely PC (16:0–13-HODE) and PC (16:0-9-HODE). Therefore, we have demonstrated a highly efficient cardioprotective effect of α-TOH through regulating oxidative stress-dependent and stress-independent properties.

Upon uptake in the liver, side chain truncation of all vitamin E forms is initiated by CYP4F2/3A4-dependent ω-hydroxylation, which forms the so-called long-chain metabolite α-13′-OH [58,59]. Subsequently, α-oxidation forms α-13′-COOH via the aldehyde metabolite processed by alcohol and aldehyde dehydrogenase. Following this, β-oxidation-induced side-chain degradation forms the intermediate-chain metabolites and short-chain metabolites, and finally the catabolic end-product of vitamin E, carboxyethyl hydroxychroman (CEHC) [60]. As shown in the work of Farley et al., subcutaneous administration of 100 mg α-TOH/kg BW in rats for 1 week resulted in accumulation of α-TOH (100 nmol/g) and α-CEHC (0.5 nmol/g) in heart tissue, with CEHC concentrations being 200-fold lower compared to α-TOH [61]. As previously described, CEHC-metabolites of vitamin E mediate anti-oxidative [62] and anti-inflammatory [63,64] effects in similar concentrations to α-TOH [63,65]. In human neutrophils, inhibition of PKC translocation and superoxide anion production induced by tocopherols and respective metabolites have been observed [62]. In addition, γ-TOH and γ-CEHC, which increased in humans as a result of supplementation with γ-TOH, have been shown to decrease plasma TNF-α and MPO concentrations [66]. Therefore, the relevance of hepatically formed

metabolites have to be considered as contributors to the anti-oxidative and anti-inflammatory effects shown in this study, as described for the vitamin analog Trolox [67]. Therefore, effects of CEHC metabolites in myocardial infarction cannot be excluded completely. Nevertheless, α-CEHC excretion may increase only after exceeding an individual α-TOH threshold (30–50 μM), depending on plasma lipid concentrations [68,69]; and is therefore a marker for (super) optimal α-TOH supply in humans [70]. Since, the aim of our study was to maintain the α-TOH concentration during I/R injury, using α-TOH supplementation of 300 mg/kg/d for 3 days, and not to generate (super) optimal conditions, the formation of CEHC and other metabolites was probably less efficient. Therefore, the contribution of α-CEHC to the observed effects is most likely less relevant for the findings in the study presented here.

The concentration of α-TOH used in our experimental setup is of central importance. The recommended daily oral intake for healthy adults is 15 mg RRR-α-TOH [71,72]. Nevertheless, even higher concentrations have been classified as safe for animals and humans. As observed by the FDA [73], α-TOH has a LD50 > 2000 mg/kg BW in mice, rabbits and rats. Several other species can tolerate oral doses of 200 mg/kg BW [74]. The European Commission Scientific Committee on Food (SCF) approved a daily oral dose of 300 mg α-TOH for humans [75]. In the human trail of Lassnigg et al., 270 mg *all-rac*-α-TOH which correlates to 180 mg RRR-α-TOH, was intravenously applied after elective cardiac surgery in humans without detecting any side effects [26]. Therefore, 300 mg α-TOH/d (5 mg/kg/d), applied intraperitoneally, has been used in our mouse study. This concentration of α-TOH is slightly higher compared to Lassnigg et al. but is still in the range of the SCF-approved daily dose, in contrast to most other studies which typically use higher doses of α-TOH [29,30]. This demonstrates that even doses of α-TOH within the approved daily intake dose are highly effective and protective in I/R injury when delivered at the crucial time around reperfusion. We hereby provide an experimental design which potentially can be translated to human trials without concern surrounding the safety of α-TOH applications.

The findings presented in our study give hope for a long sought-after therapy for I/R injury, but clearly need to be confirmed in human patients. α-TOH has been extensively tested as a preventive drug with the aim of reducing the rate of cardiovascular events, including MI. However, this approach has been reported to be inefficient, as confirmed by the outcomes of several human intervention and correlation reports published indicating a beneficial effect of vitamin E in cardiac ischemia/reperfusion [29,30,38]. Yau et al. reported that pre-treatment with α-TOH before elective cardiopulmonary bypass showed improvement in myocardial metabolism and ventricular studies [38]. This finding reflects the effects of α-TOH on chronic atherosclerosis, particularly plaque instability and the risk of plaque rupture. However, oxidative stress as the main target for vitamin E therapy will clearly be increased to a much higher level in acute I/R injury compared to chronic atherosclerosis. The potential beneficial effects of α-TOH on infarct size and preservation of cardiac function in MI have been overshadowed and have not been thoroughly investigated. This is based on the negative outcomes of the above-mentioned clinical trials testing of vitamin E for its capability to reduce the cardiovascular event rate. This is surprising as there were a few early function, unfortunately with no clinical significance [38]. Although these reports had limitations such as using *ex vivo* heart preparations, non-pharmacological doses or limited scope of data and mechanistic work up, these reports indicated the value of a further thorough study. The outcome of our study is noteworthy in the extent of cardio protection shown by vitamin E and the mechanistic insight provided. However, our encouraging preclinical data need to be confirmed in clinical trials with patients presenting with ST-elevation MI, and the use of cardiac enzymes and echocardiography or magnetic resonance imaging to assess α-TOH's potential to preserve cardiac function in patients.

Our study sheds new light on the potential of the acute therapy with α-TOH in patients presenting with MI, and may ultimately offer an effective low-cost treatment for the many patients suffering a MI. α-TOH in a dose of up to 300 mg/d, a concentration equivalent to the one used in our study, is already approved by the SCF and considered safe in humans without adverse effects. This dose would be attractive and highly feasible for the treatment of MI patients and ultimately has the potential to provide a better outcome for patients suffering a MI. As there is currently no drug available in the clinic that can reduce the cardiac damage caused by I/R injury, the potential impact on cardiovascular health would be significant. We postulate that α-TOH therapy/supplementation compensates for the drop seen during I/R injury and would facilitate an adequate anti-oxidative defense within the ischemic and reperfused myocardium. Ultimately, α-TOH holds promise as an inexpensive and readily translatable novel treatment preventing cardiac damage and thereby reducing mortality and morbidity in patients who suffer a MI.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101292.

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7.6 Manuscript VI

Chapter 9

Garcinoic Acid: A Promising Bioactive Natural Product for Better Understanding the Physiological Functions of Tocopherol Metabolites

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Chapter Outline

Introduction **436**
Garcinia kola **437**
Bioactive Ingredients of the *Garcinia kola* Nut 438
Biflavones and Benzophenone Derivatives 439
Garcinal 439
Garcinoic Acid 441
Vitamin E **442**
Biological Significance of Vitamin E 443
Absorption, Transport, and Distribution of Vitamin E 446
Metabolism of Vitamin E 446
Synthesis of Vitamin E Long-Chain Metabolites **448**
Isolation of Garcinoic Acid 449
Synthesis of Garcinoic Acid 451
Semisynthesis of Long-Chain Metabolites From Garcinoic Acid 452
Bioactivity of Garcinoic Acid, Vitamin E, and Long-Chain Metabolites **454**
Cytotoxicity 455
Cytotoxic Effects of Vitamin E 455
Metabolites of Vitamin E 456
Garcinoic Acid 457
Antioxidative Properties 458
Antiinflammatory Actions 461

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Cyclooxygenases and Their Lipid Mediator Products 461
Vitamin E Modulates Prostaglandin E_2 Release and Cyclooxygenase Activity 462
Effect of Long-Chain Metabolites of Vitamin E on Cyclooxygenase 2 Expression 465
Vitamin E and Lipoxygenases 466
Modulation of Lipid Homeostasis 468
Tocopherols and Macrophage Foam Cell Formation 469
Effects of Long-Chain Metabolites and Garcinoic Acid on Macrophage Foam Cell Formation 470
Conclusions and Perspectives 471
Abbreviations 473
Acknowledgments 475
References 475

INTRODUCTION

Organisms produce bioactive natural products (secondary metabolites) as an adaption to their environment or as defense mediators. In contrast to primary metabolites such as protein, fat, and carbohydrates, they are not essential for growth, development, or reproduction [1,2]. Nevertheless, secondary metabolites are, like no other compounds, representatives for medical progress and have enormous importance for human health care. The use of natural products as medicines developed over generations and has been described throughout history in the form of folk medicine. The traditional African, Korean, Chinese, Islamic, and herbal medicines are the most important forms of historical folk medicine. Especially in Africa and Asia, 80% of the population still relies on traditional medicine for their primary health needs [3]. In these regions, fungi, plants, marine algae, or marine sponges are the most popular sources for bioactive natural products, but many of these compounds remain unexplored [2]. Nevertheless, plants are the dominant source of natural products in folk medicine. Plants have been well documented for their medicinal use for several thousands of years [4]. A well-known example is the plant *Alhagi maurorum*, which was used by the Romans for treating nasal polyps [5]. Plant-based traditional medicine was very important for primary health care over hundreds of years, but during the 18th century, the understanding of medicine changed. After Leeuwenhoek identified the first microorganism, enormous progress in the prevention of diseases was made. The knowledge associated with traditional medicine has promoted further investigations of compounds and extracts obtained from medicinal plants as potential medicines. This led to the isolation of many natural products from different sources.

One of the most famous examples is the antiinflammatory agent acetylsalicylic acid (aspirin) derived from the natural product salicin, which was isolated from the bark of the willow tree *Salix alba* [6,7]. During this

period, ethnopharmacological knowledge has been used for early drug discovery. Today, advances in analytical technologies improve the discovery of new bioactive natural products. These compounds have unique structural properties in comparison to products from standard combinatorial chemistry, making them the most promising source of lead structures for drug development [8,9].

A good example for the development from a medical plant used in traditional African medicine to a source of bioactive products for putative drugs is the African plant *Garcinia kola*. The parts of this plant contain many bioactive compounds, including the δ-tocotrienol (δ-T3) derivate garcinoic acid, which comprises an interesting molecule for functional studies. The aim of this review is to summarize the knowledge on this promising molecule and its use in research on vitamin E and its metabolites.

GARCINIA KOLA

G. kola or bitter kola is a dicotyledonous plant of the family *Clusiacea* (Fig. 9.1). It can be found in the rain forests of west and central Africa where it grows as a medium-sized tree with a height up to 12 m [10,11], but *G. kola* is also used for commercial farming, especially in Nigeria. The plant has reddish fruits containing two to four seeds. Both fruit components can be eaten [12]. *G. kola* plants bloom between December and January and their fruits mature from June to August [13]. From the botanical point of view, the fruits belong to the class of berries, but the seeds are often called *G. kola* nuts [14]. The nuts are dried and afterward available over the whole year, which gives them a small economical relevance [12]. Because of the bitter flavor of its seeds, the plant is colloquially called "bitter kola" or "bitter nut." The locals also name it "Orogbo" (Yoruba), "Aku ilu" (Igbo), and "Namijin goro" (Hausa) [3].

Apart from its small economical relevance, *G. kola* is very important for African ethnomedicine. Approximately 60–80% of the African population depend on herbal cures for their primary health care [3]. In the traditional African medicine, each part of the *G. kola* plant is used for different medical applications. For example, the root is used for oral hygiene and the tree bark as an abstergent agent. The latex of the tree is put on fresh wounds to prevent septic inflammation and to support healing [15]. The nuts are used for treating bronchitis and infections of the pharynx and colic [10]. Furthermore, the nuts are also used as antivenom for people with suspected intoxication [16]. It is also speculated that *G. kola* nuts protect against the toxic effects of alcohol [17]. Because of their bitter flavor, the bitter nuts are also used as stimulants for inducing anorexia [15]. Furthermore, antimicrobial effects [18–20], antiviral effects [10], antiparasite effects [21], antidiabetic effects [22], and hepatoprotective effects [23] have been described.

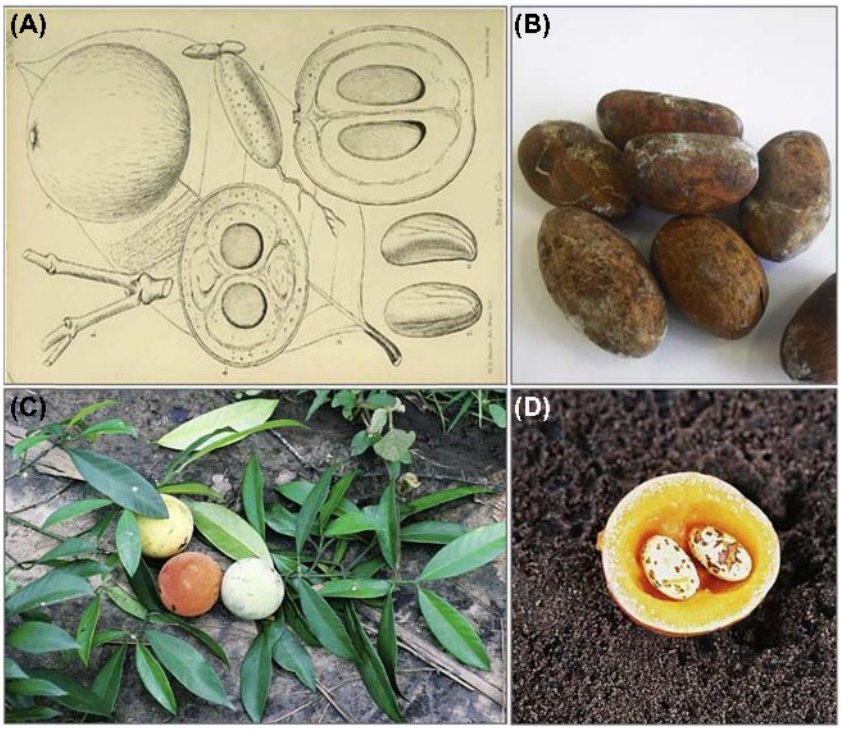

FIGURE 9.1 (A) Botanical illustration of the fruits of *Garcinia kola* E. Heckel *(Drawing by W.G. Smith published in 1875.)*. The fruits are shown completely and in cross section. Further, the seeds, colloquially called "bitter nut," can be seen. The illustration is entitled "bitter nut." (B) Photography of *G. kola* seeds. (C) Photograph of *G. kola* plant with fruits *(By courtesy of Paul Latham.)*. (D) Cross section of *G. kola* fruit, showing seeds *(By courtesy of Paul Latham.)*.

Bioactive Ingredients of the *Garcinia kola* Nut

The main components of *G. kola* nuts are carbohydrates, protein, fiber, fat, and water [3,12]. In contrast to the real kola nuts (*Cola nitida*), the bitter nuts do not contain caffeine [17], but they are a good source for calcium, potassium, sodium, and magnesium [3,24]. Furthermore, many other bioactive compounds, including tannins, saponins, alkaloids, and glycosides, have been isolated from *G. kola* nuts [3,13]. The nut also contains flavonoids and benzophenone derivatives such as kolaflavones and *Garcinia*-biflavones 1 (3,4,4,5,5,7,7-heptahydroxy-3,8-biflavanone) and 2 (3,4,4,5,5,5,7,7-hexahydroxy-3,8-biflavanone), which might be responsible for the observed antimicrobial effects of *G. kola* nuts [3]. Furthermore, two chromanols, garcinal and garcinoic acid, which have been described as strong antioxidants, have been isolated from *G. kola* seeds [25].

Biflavones and Benzophenone Derivatives

Most of the biochemical and physiological effects of the *G. kola* nut are attributed to its content of biflavones and benzophenone derivatives. One of the most investigated nut biflavones is kolaviron, a dimeric flavonoid (Fig. 9.2). In addition to its hepatoprotective effects [22] and its ability to lower blood cholesterol [26], antiinflammatory capacity has been shown for this compound in different animal models. For example, diabetic rats were supplemented with 100 mg/kg kolaviron for 6 weeks. The treatment with kolaviron resulted in a reduction of inflammatory processes, indicated by reduced serum concentrations of interleukin (IL)-1β and monocyte chemotactic protein 1 (MCP1) [27]. Similar results have been found in hepatic tissues of diabetic rats, where treatment with kolaviron reduced the amount of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor α (TNFα) [28]. Further studies investigated the effects of kolaviron on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) 2 expression in hepatic tissues of dimethylnitrosamine-treated rats. Dimethylnitrosamine is known as a hepatotoxin that enhances expression of iNOS and COX2 proteins as part of the proinflammatory response. After treatment with kolaviron, a significant reduction of dimethylnitrosamine-upregulated iNOS and COX2 expression was measured, indicating that kolaviron acts as an antiinflammatory factor. In addition, electrophoretic mobility shift assays showed that this effect may result from reduced formation of the transcription factors nuclear factor "kappa-light-chain-enhancer" of activated B cells (NFκB) and activator protein 1 (AP-1) [29]. Furthermore, interactions of kolaviron with several intracellular immune mediators, such as IL-1α, IL-1β, IL-18, and IL-33, have been observed in murine RAW264.7 macrophages. In this context, kolaviron has been shown to modulate expression and phosphorylation of proteins involved in NFκB, mitogen-activated protein kinase, AP-1, and protein kinase B (PKB/Akt) signaling, leading to an inhibition of the lipopolysaccharides (LPS[1])-induced immune response [30].

Garcinal

The isolation procedure from *G. kola* not only provides garcinoic acid and δ-T3[2], but also garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H,2-chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al] [25]. This structure is closely related to garcinoic acid; solely an aldehyde moiety terminates the side chain instead of a carboxylate moiety (Fig. 9.3).

1. Lipopolysaccharides are endotoxins composed of lipid and polysaccharide components found in gram-negative bacteria that provoke strong immune responses in eukaryotes.
2. Tocotrienols are composed of a chroman ring system and an unsaturated side chain; they constitute a subgroup of vitamin E (the reader is referred to the section "Vitamin E").

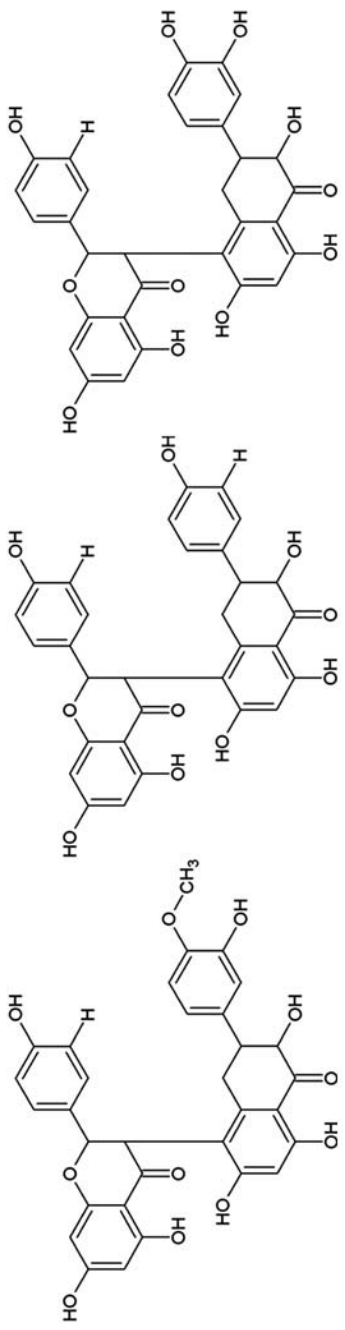

FIGURE 9.2 Chemical structures of biflavones of *Garcinia kola*. *Adapted from O.A. Adaramoye, V.O. Nwaneri, K.C. Anyanwu, E.O. Farombi, G.O. Emerole, Clin. Exp. Pharmacol. Physiol. 32 (2005) 40–46.*

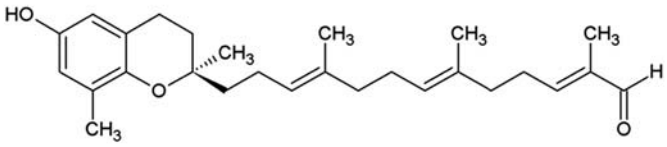

FIGURE 9.3 Chemical structure of garcinal [13-(6-hydroxy-2,8-dimethyl-3,4-dihydro-2H, 2-chromenyl)-2,6,10-trimethyl-2,6,10-tridecatrien-1-al]. *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619 1625.*

However, the metabolic pathways leading to the formation of garcinal in plants have not been elucidated. Based on the structural similarity to garcinoic acid, garcinal likely has a comparable bioactive potential. Nevertheless, the bioactive properties of garcinal are largely unknown. To the best of our knowledge, merely two works addressed the effects of the isolated compound. According to these, garcinal is 1.5 times more potent than α-tocopherol (α-TOH[3]) and has a similar antioxidative activity as garcinoic acid as well as δ-T3 [25]. Furthermore, replacing the terminal functional group of the side chain of garcinoic acid (or garcinal respectively) does not alter the antioxidative capacity [25]. These findings support the hypothesis that garcinoic acid and garcinal may have similar properties in biological systems. Although the health-promoting effects of extracts from *G. kola* (vide supra) are generally ascribed to the biflavones, garcinoic acid and garcinal should be taken into account. This became evident when different fractions of the crude extract were examined regarding their antioxidant and radical-scavenging activities. It turned out that the most potent fraction contained the *Garcinia* biflavone 1 and 2 but also garcinoic acid and garcinal [31]. Given the antioxidative potential of the isolated chromanols, garcinoic acid and garcinal likely contribute substantially to the effects of extracts from *G. kola*. Garcinal is therefore an interesting compound for functional studies due to its structural properties and for explaining the health-promoting effects of *G. kola*.

Garcinoic Acid

Garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol) is an interesting δ-T3 derivative and its occurrence in *G. kola* nuts was first described by Terashima and coworkers in 1997 [32]. A few years later, the same group published a method for the isolation of garcinoic acid from *G. kola* nuts [25]. However, *G. kola* nuts are not the only source of δ-tocotrienolic acid. The extraction of garcinoic acid from members of the *Clusiaceaen* plant family [33] and the development of a stereo-controlled synthesis [34] have been described. For an explicit description of the isolation and synthesis of garcinoic acid, the reader is

3. Tocopherols are characterized by a chroman ring system and a saturated side chain; they constitute a subclass of vitamin E (the reader is referred to the section "Vitamin E").

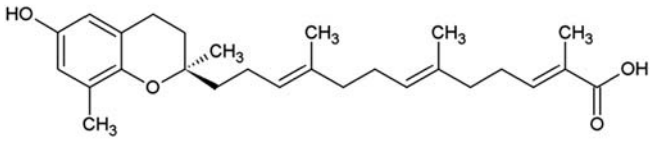

FIGURE 9.4 Chemical structure of garcinoic acid (*trans*-13′-carboxy-δ-tocotrienol). *Adapted from K. Terashima, Y. Takaya, M. Niwa, Bioorg. Med. Chem. 10 (2002) 1619 1625.*

referred to the section "Synthesis of Vitamin E Long-Chain Metabolites." Garcinoic acid is in principle a metabolite of δ-T3 with the carboxylic group placed at the end of the aliphatic side chain (Fig. 9.4), which would be formed in humans in the liver after dietary intake of δ-T3. Thus, garcinoic acid shares structural similarities with δ-T3 [33].

Garcinoic acid shows many bioactive properties. The high antioxidant potential is probably one of the best investigated ones [25,31]. Furthermore, antiproliferative effects were shown in carcinoma cells by Mazzini et al. [33]. The acid also acts as a DNA polymerase β inhibitor, indicating that garcinoic acid is able to disturb base excision repair in tumor cells [34]. This finding supports the results of Mazzini and coworkers. For an explicit description of the bioactive properties of garcinoic acid, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites."

Because of its high content of bioactive components, the *G. kola* nut has great potential for pharmaceutical applications, which is reflected by a number of patents. In 1987, the first patent for a biflavone isolated from *G. kola* as an ingredient for the treatment of liver diseases was registered [35]. The natural product reduced hepatocyte damage in a galactosamine-treated rat model of acute hepatotoxicity and improved liver values in patients with hepatitis [35]. Furthermore, an extract containing a mixture of different biflavones (*Garcinia* biflavones 1, 1a, and 2 as well as kolaflavones) of *G. kola* is used as an antiglycation agent and is also registered in a patent [36]. This compound lowers the accumulation rate of advanced-glycation adducts in the human body; high concentrations of these adducts can damage cells and tissues [36]. The existing patents on bioactive compounds of *G. kola* for the use as pharmaceuticals provide evidence for the growing interest in this plant [35,36]. The role of *G. kola* as an important part of the African ethnomedicine evolved to an interesting source of natural compounds for modern drug development. Although only patents on biflavones have been registered to date, garcinoic acid is also a promising lead compound for future pharmaceuticals.

VITAMIN E

Vitamin E is naturally found in a variety of plant products, such as oils, nuts, germs, seeds, and in smaller quantities in vegetables and some fruits. Due to their lipophilic character, the several molecules summarized as "vitamin E"

are associated to fats in dietary sources. In fact, vitamin E is a hypernym for different molecules, which can be classified as TOH, T3, and a less consistent group of vitamin E–related structures (Fig. 9.5). The common feature of all molecules is the chroman ring and a covalently connected phytyl-like side chain, whose respective constitutions define the individual vitamin E forms. Characteristic for the TOH is their saturated side chain, whereas T3 carry three double bonds in this substructure. The methylation pattern of the chroman ring determines the classification as α-, β-, γ-, or δ-form of the TOH or T3, respectively. More precise, besides position 8, positions 5 and 7 are crucial: α means methylation at position 5, 7, and 8, β at position 5 and 8, γ at position 7 and 8, and δ solely at position 8 of the chroman ring. Natural forms of vitamin E exist in the RRR configuration (TOH) or the R configuration (T3), whereas synthetic vitamin E is a mixture of the different stereoisomers. Members of the group of the vitamin E–related structures can either be more similar to TOH, such as tocomonoenol or marine-derived TOH, or to T3, such as desmethyl-(P_{21})T3, desmethyl-(P_{25})T3, and plastochromanol-8 (Fig. 9.5).

Biological Significance of Vitamin E

Although it is controversially discussed how vitamin E benefits human health, it is an essential factor, as the classification as a vitamin shows. Vitamin E was discovered in 1922 as vital for the fertility of rats [36a], but is also essential for the maintenance of human health. Several disease states have been linked to vitamin E deficiency. A severe effect of inadequate vitamin E supply is anemia. Vitamin E is known for its strong antioxidative properties; if these are lost, erythrocytes are prone to rupture due to higher fragility of their cell membrane [37]. Based on this observation, erythrocyte hemolysis was used as a biomarker to set the recommended daily allowance of 15 mg per day for adults [37]. Not only erythrocytes, but also components of the nervous system are negatively affected by vitamin E deficiency. An isolated vitamin E deficiency, i.e., a deficiency not caused by fat malabsorption, characterizes "ataxia with vitamin E deficiency."[4] This disease is caused by defects in the gene encoding for the α-TOH transfer protein, namely *TTPA*, leading to an impaired ability to retain α-TOH and to depleted α-TOH plasma levels [38,39]. Likely due to the loss of antioxidant protection, nerve cells degenerate and neurological symptoms such as ataxia, dysarthria, hyporeflexia, and decreased vibration sense occur [40].

Vitamin E deficiency might also occur due to fat malabsorption, for example, caused by cystic fibrosis or some liver diseases as well as genetic

4. Ataxia with vitamin E deficiency is an autosomal recessive disorder characterized by markedly reduced plasma levels of vitamin E, ataxia (neurological symptom with a lack of voluntary coordination of muscle movements), spinocerebellar degeneration, and peripheral neuropathy that resembles Friedreich ataxia.

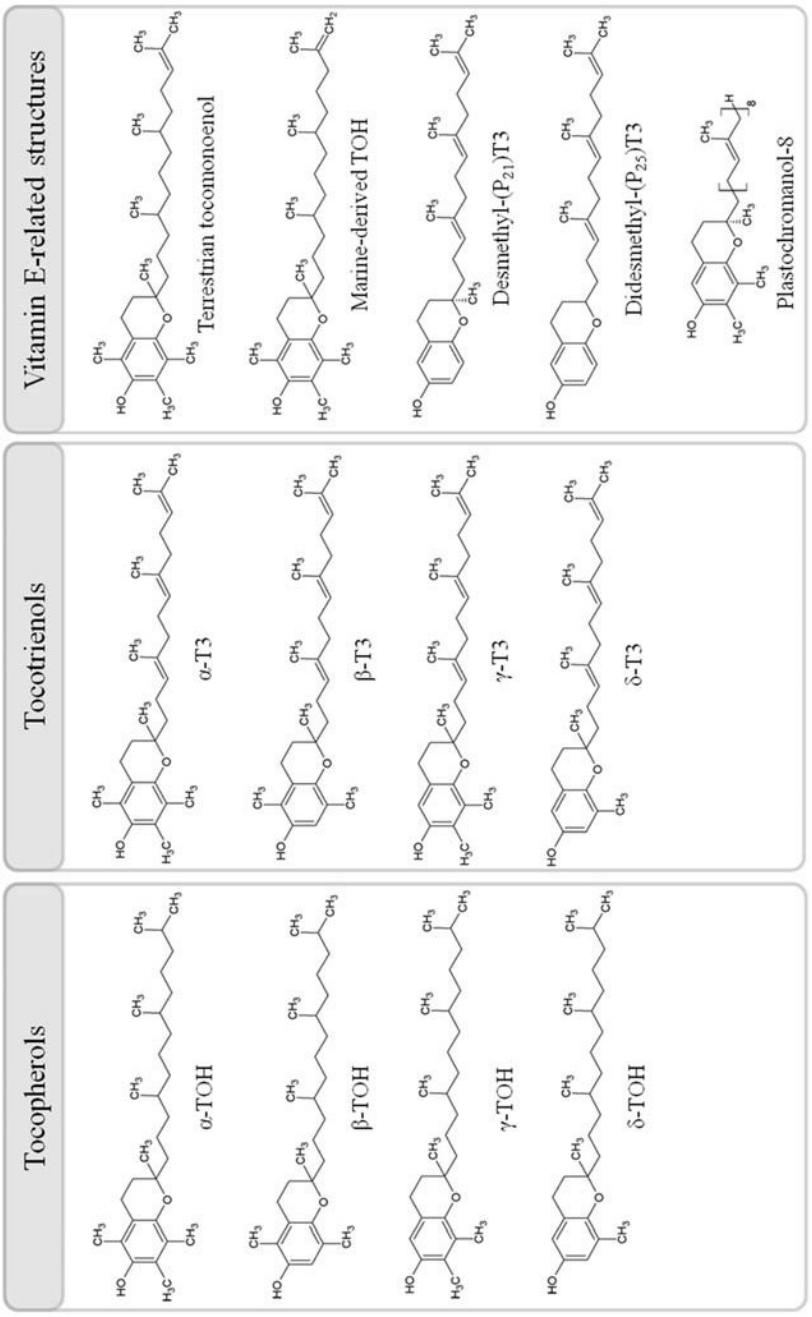

FIGURE 9.5 Chemical structures of vitamin E forms and vitamin E related natural compounds.

defects, such as abetalipoproteinemia [41–43]. Further, the Marinesco-Sjögren syndrome and chylomicron retention disease likely cause vitamin E deficiency, as they are characterized by impaired chylomicron assembly or delivery [44,45]. Consequently, peripheral nerves die due to the lack of vitamin E, leading to spinocerebellar ataxia. Long-term vitamin E deficiency is further characterized by muscle degeneration. This process can ultimately lead to death if the heart muscle is affected [46]. Given its protective role on neurons, vitamin E was expected to prevent age-related neurodegenerative diseases such as Alzheimer disease. Indeed, vitamin E supplementation slowed down the progression of Alzheimer disease in some human intervention trials [47,48]. Supportive findings were also made in mice, where vitamin E deficiency caused axonal degeneration in brain areas important for memory and cognition [49]. Furthermore, impaired motor coordination and cognitive function was normalized by supplementation with vitamin E in vitamin E–depleted mice [50].

Vitamin E status seems to be important not only for the maintenance of neurons, but also for their development. Several animal studies suggest that the sufficient supply with vitamin E (of the mother) is critical for the development of the central nervous system and cognitive function of the offspring [51–53]. Furthermore, vitamin E along with folic acid may play a supportive role in the prevention of neural tube defects in human [54,55].

For a long time, the effects of vitamin E were attributed to its antioxidant properties (vide supra), but more recent work was dedicated to its non-antioxidant properties. Hence, it became evident that vitamin E modulates gene expression and enzyme activities and interferes with signaling cascades independent of its antioxidative capacity [56]. Examples for such functions are the suppression of inflammatory mediators, reactive oxygen species (ROS[5]), and adhesion molecules; the induction of scavenger receptor; and the activation of NFκB [57]. Given these (and further known) actions, vitamin E is most likely playing a role in several, but not only, inflammatory diseases (for more details, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). In addition, T3—another relevant form of vitamin E in our diet—are gaining more attention. Neuroprotective, anticancerogenic, antidiabetic, and cardioprotective effects have been suggested for this group of vitamin E [58]. However, further research is required, as the results obtained from clinical trials for TOH are inconsistent with respect to beneficial effects on chronic diseases such as cancer and cardiovascular diseases (CVD[6]) [59].

5. Reactive oxygen species are oxygen-containing molecules that are highly reactive, such as superoxides, peroxides, hydroxyl radicals, and singlet oxygen.
6. Cardiovascular diseases comprise disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, pulmonary embolism, and others.

Absorption, Transport, and Distribution of Vitamin E

Vitamin E comprises a class of lipophilic molecules and hence its intestinal uptake follows the pathway known for lipids. A key step is the lipid emulsification, i.e., the incorporation into micelles formed with the help of phospholipids and bile acids. The transfer into enterocytes of the intestine is carried out by passive diffusion, scavenger receptor class B type 1 (SRB1) [60], or Niemann-Pick C1-like protein 1 [61]. As there are no specific transport plasma proteins known for α-TOH [62], it is assumed that vitamin E transport in blood follows that of lipoproteins (reviewed in Ref. [61]). Here, key players in the uptake of vitamin E are SRB1 in peripheral tissue and low-density lipoprotein (LDL) receptor as well as LDL receptor–related protein in the liver [63,64]. Once in the liver, discrimination between the different forms of vitamin E occurs. Responsible for this process is the α-tocopherol transport protein (α-TTP), which promotes the incorporation of *2R*- or *RRR*-α-TOH into very-low-density lipoproteins (VLDL) [65,66], whereas other forms and stereoisomers are secreted into bile [67]. Besides α-TTP, the TOH-associated protein and the TOH-binding protein are known mediators of the intracellular transport of vitamin E. Interestingly, α-TOH secretion from the liver is apparently not dependent on VLDL assembly and secretion, thus oxysterol-binding proteins [68] and ATP-binding cassette transporter A1 (ABCA1) [69] have been suggested to contribute to the release from the liver. Furthermore, ABCA1 mediates the efflux of vitamin E in the intestine, macrophages, and fibroblasts [69], and multidrug resistance P glycoprotein has been identified as a transporter for the excretion of α-TOH via bile [70].

Metabolism of Vitamin E

The metabolism of vitamin E mainly takes place in the liver, whereas extrahepatic pathways have also been suggested [71,72]. Interestingly, rates of vitamin E metabolism increase with higher levels of the vitamin to prevent its accumulation to toxic levels. As indicated before, the preferred form of vitamin E in humans is α-TOH, which is due to the preferential binding of a specific hepatic protein, namely α-TTP. It has been hypothesized that α-TTP protects the α-form from metabolism, in turn leading to its enrichment. Given the lower affinities of the other vitamin E forms to α-TTP, their rate of catabolism is likely more pronounced [73]. In principle, metabolism of all forms of vitamin E follows the same route, which was confirmed by the detection of the respective end products of hepatic metabolism, α-, γ-, and δ-carboxyethyl-hydroxychromanol (CEHC) (Fig. 9.6) [74,75]. However, the catabolic rates depend on the vitamin E form, possibly due to different affinities to key enzymes [73,76]. The classification of the metabolic end product as α-, γ-, and δ-CEHC indicates that the chroman ring is not modified in this process; the aliphatic side chain is rather the substructure

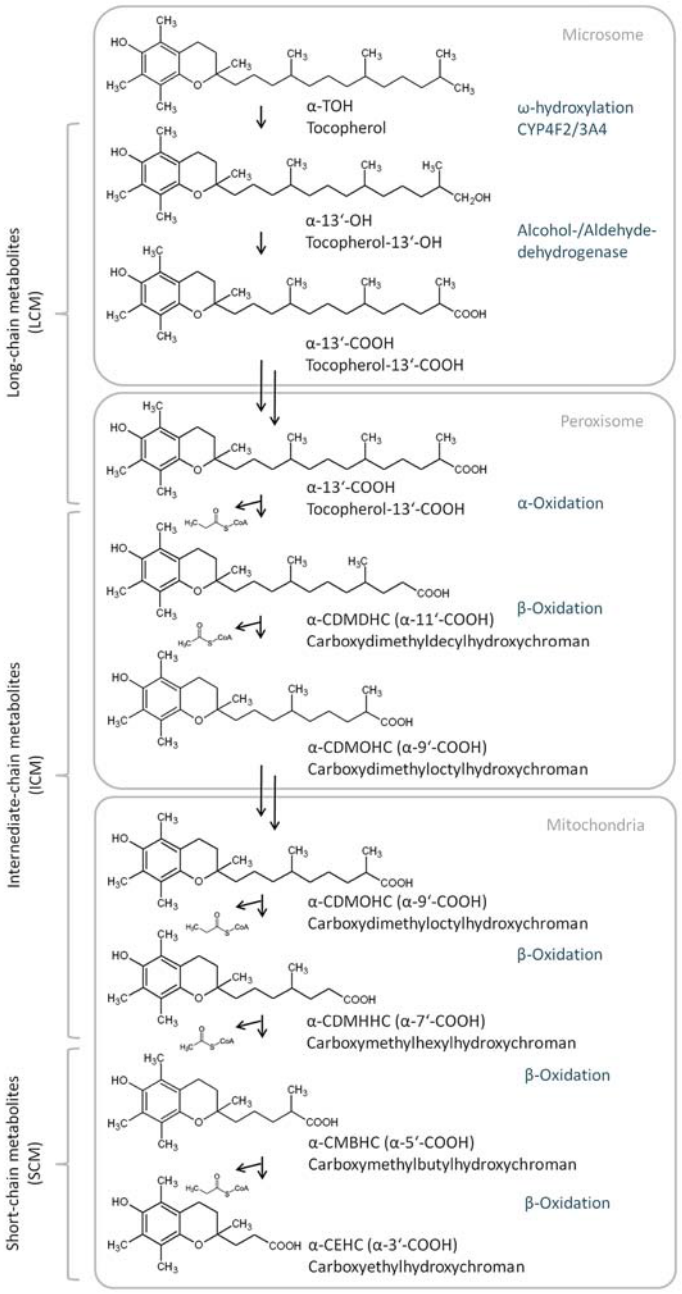

FIGURE 9.6 Principle hepatic metabolism of vitamin E. *Adapted from M. Birringer, P. Pfluger, D. Kluth, N. Landes, R. Brigelius-Flohé, J. Nutr. 132 (2002) 3113 3118.*

where modification takes place. The same applies to T3, whereas further enzymes such as 2,4-dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase (known from the metabolism of linoleic acid) are likely needed for metabolizing the unsaturated side chain [77].

Metabolism of vitamin E is therefore characterized by the shortening of the side chain *au fond*. Catabolism of the vitamin E molecule takes place in three cell compartments: the endoplasmic reticulum (microsomes), peroxisomes, and mitochondria. However, the transfer of the metabolites between the compartments is not yet understood. The initial step takes place at the endoplasmic reticulum and results in the formation of 13′-hydroxychromanol (13′-OH) metabolites via ω-hydroxylation by cytochrome P450 (CYP) 4F2 or CYP3A4, respectively [76,78]. Subsequent ω-oxidation by alcohol and aldehyde dehydrogenase (an aldehyde intermediate is formed) leads to 13′-COOH metabolites. Hence, the metabolites are handled like fatty acids and the side chain is shortened by β-oxidation, resulting in the elimination of propionyl-CoA or acetyl-CoA, respectively. The first two rounds take place in the peroxisome, leading to the intermediate-chain metabolites 11′-COOH and 9′-COOH, respectively. Three further rounds of β-oxidation are carried out in the mitochondria, forming the short-chain metabolites (SCM) 7′-COOH and 5′-COOH as well as the final product CEHC or 3′-COOH. During catabolism, the metabolites are modified simultaneously by conjugation, i.e., the metabolites are either sulfated or glucuronidated, but glycine-, glycine-glucuronide-, and taurine-modified metabolites have also been identified [79]. The more hydrophilic conjugated SCM are released via urine. In human urine, however, vitamin E is mainly found in conjugated form after glucuronidation [75,80–82]. The long-chain metabolites (LCM[7]) and their metabolic precursors are secreted via bile into the intestine. This fecal route is considered as the major way of excretion for vitamin E. In contrast to urine, the metabolites in fecal samples are not conjugated [80,83].

SYNTHESIS OF VITAMIN E LONG-CHAIN METABOLITES

The LCM can be obtained in vitro by incubation of cultured cells with the respective TOH precursors (the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites"). The culture supernatants of these cells can be used to investigate the cellular effects of the LCM or their action on isolated enzymes, as it has been already practiced by Jiang et al. [84]. However, this method is not feasible for all investigations, as the cells produce a mixture of carboxychromanols with different chain lengths, including SCM, as well as sulfated and nonconjugated metabolites. Furthermore, not all cell types exhibit the capability to metabolize all forms of TOH

7. The long-chain metabolites of vitamin E are the metabolites of tocopherols and tocotrienols with a side chain that is comprised of 13 carbon atoms.

[85,86]. A purification of defined metabolites is therefore needed if one is interested in investigating the specific effects of a single metabolite.

An alternative way to obtain pure metabolites is their chemical (semi) synthesis. The semisynthesis of α- and δ-13′-OH and the respective 13′-COOH metabolites has been established using the natural product garcinoic acid [33,87]. The first step in the entire process is the extraction (or synthesis) of garcinoic acid from appropriate sources, which is described in the following section. The subsequent synthesis of the α- and δ-LCM from garcinoic acid is outlined in another section.

Isolation of Garcinoic Acid

The isolation of garcinoic acid was first mentioned in 1984 by Franco Delle Monache and colleagues, who used *Clusia grandiflora* from Venezuela as source material [88]. In general, the family of *Clusiaceae* is the source of choice for isolating garcinoic acid. The *Clusiaceae* family is comprised of about 40 genera including about 1600 species, which are found in tropical regions worldwide [89–91]. Members of the family are sources of, inter alia, edible fruits, drugs, pigments, and dyes [90] and have therefore been used in traditional medicine in the regions of their occurrence [89]. So far, three genera of the *Clusiaceae* are known to contain garcinoic acid, namely *Tovomitopsis*, *Clusia*, and *Garcinia*. An overview of reported isolation procedures is provided in Table 9.1.

Tovomitopsis psychotriifolia, a plant from Costa Rica, has been shown to contain garcinoic acid in its leaves. In 1995, Setzer et al. extracted the compound from fresh chopped leaves using 80% aqueous ethanol with a subsequent isolation by liquid chromatography and thin-layer chromatography (TLC) using a 1:1 ethyl acetate/hexane mixture. Determination of the structure was carried out by nuclear magnetic resonance (NMR). Here, the detected structure was *trans*-δ-tocotrienolic acid, whereas Monache et al. mainly found the *cis*-isomer [92].

Among the *Clusia* genus, several members produce garcinoic acid. The trunk of Brazilian *Clusia obdeltifolia* contains a mixture of garcinoic acid in its *cis*- and *trans*-configuration. Extraction of the compounds from dried and powdered material was carried out by hexane with subsequent evaporation of the solvent [93]. Following fractionation with ethyl acetate/hexane and hexane/acetone on a silica column led to the isolation of garcinoic acid. Here, the *cis*-form was more prominent than the *trans*-form with an approximate ratio of 9 to 1, as determined by NMR [93]. The related plant *Clusia burlemarxii*, found in Brazil, also contains garcinoic acid in its leaves. The natural product was extracted from the dried and powdered material by maceration with 95% ethanol, concentration, mixing with 80% ethanol and subsequent treatment with ethyl acetate. Garcinoic acid was then purified by column chromatography over silica gel with mixtures of

TABLE 9.1 Overview of Procedures for Garcinoic Acid Isolation

Plant	Source	Method[a]					Refs.
		Extraction	Separation Process		Input	Yield	
Tovomitopsis psychotriifolia	Leaves	EtOH	LC, TLC	HEX/AcOH	0.16% of starting weight		[92]
Clusia obdeltifolia	Trunk	HEX	CC	**1.** EtAc/HEX **2.** HEX/ACE	6 kg	1.512 g	[93]
Clusia burlemarxii	Leaves	**1.** EtOH **2.** EtAc	CC	**1.** TCM/MeOH **2.** AcOH/MeOH	1.6 kg	5 mg	[89]
Clusia pernambucensis	Bark	EtAc	CC, TLC	**1.** cHEX/EtAc **2.** EtAc/MeOH	197 g	85.3 mg	[94]
			HPLC	H_2O/MeOH/ACN			
Garcinia kola	Seed	**1.** MeOH **2.** MeOH/TCM	CC	**1.** MeOH/TCM **2.** HEX/ACE	1 kg	3.8 g	[87]
Garcinia amplexicaulis	Bark	**1.** DCM **2.** MeOH	CPT	HEP/EtAc/ MeOH/H_2O	270 g	10 mg	[95]

ACE, acetone; *ACN*, acetonitrile; *AcOH*, acetate; *CC*, column chromatography; *cHEX*, cyclohexane; *CPT*, centrifugal partition chromatography; *DCM*, dichloromethane; *EtAc*, ethyl acetate; *EtOH*, ethanol; *HEP*, heptane; *HEX*, hexane; *HPLC*, high-performance liquid chromatography; *LC*, liquid chromatography; *MeOH*, methanol; *TCM*, chloroform; *TLC*, thin-layer chromatography.
[a]*For detailed information, the reader is referred to the text.*

chloroform and methanol in increasing polarity and in a second washing step with mixtures of ethyl acetate and methanol in increasing polarity. Again, the *cis*-isomer was more prominent [89]. A third member of the family, *Clusia pernambucensis* from Brazil, contains garcinoic acid in the bark [94]. The extract was obtained by maceration with ethyl acetate and subsequently fractionated by column chromatography with a cyclohexane/ethyl acetate gradient and sequentially an ethyl acetate/methanol gradient. After profiling with TLC, the appropriate fraction was purified by reverse-phase high-performance liquid chromatography (HPLC) using an isocratic 8:32:60 mixture of water, methanol, and acetonitrile. In addition to the *cis*-isomer of garcinoic acid, the related compounds δ-T3, δ-T3 alcohol, and δ-T3 methyl ester were obtained. However, in terms of quantity, garcinoic acid was substantially more abundant than the other compounds [94].

Members of the genus *Garcinia* are another valuable source of garcinoic acid. The isolation of garcinoic acid from seeds of *G. kola*, which originate from Nigeria, was first described by Terashima et al. in 1997 [25,96]. Based on this procedure, Birringer et al. developed a modified method [87]. Here, the mashed seeds were extracted with methanol, and after evaporation of the solvent, the extract was dissolved in a 95:5 mixture of methanol and chloroform. The crude extract was obtained by drying. For the isolation of garcinoic acid, the extract was again dissolved in 95:5 methanol/chloroform and applied to a silica gel column for purification. Further chromatographic separation on silica gel with a 65:35 mixture of hexane and acetone led to purified garcinoic acid, as characterized by NMR and mass spectroscopy (MS) [87]. A further member, *Garcinia amplexicaulis* from New Caledonia, contains garcinoic acid in the bark. Extraction of garcinoic acid from dried and grounded material was carried out with dichloromethane and subsequently methanol in a Soxhlet apparatus. The extract was further fractionated with a 2:1:2:1 mixture of heptane, ethyl acetate, methanol, and water using centrifugal partition chromatography. Garcinoic acid was subsequently isolated from the appropriate fraction by preparative HPLC using methanol and determined by NMR and MS [95].

Synthesis of Garcinoic Acid

With the first isolation and description of garcinoic acid (δ-*trans*-tocotrienolic acid) from *Clusia grandiflora*, the groundwork for approaches to synthesize this bioactive compound was laid. In 2005, David Maloney and Sidney Hecht reported a procedure to synthesize garcinoic acid (Fig. 9.7).

The basis for their stereo-controlled synthesis was to elaborately produce two molecules: alkyl iodide, (*S*)-1-iodo-5-(2,5-dimethoxy-3-methylphenyl)-3-methylpentan-3-ol (**4**), and vinyl iodide, (2*E*,6*E*,10*E*)-ethyl 11-iodo-2,6,10-trimethylundeca-2,6,10-trienoate (**5**). The alkyl iodide (**4**) was synthesized in two reaction steps from 4-(2,5-dimethoxy-3-methylphenyl)

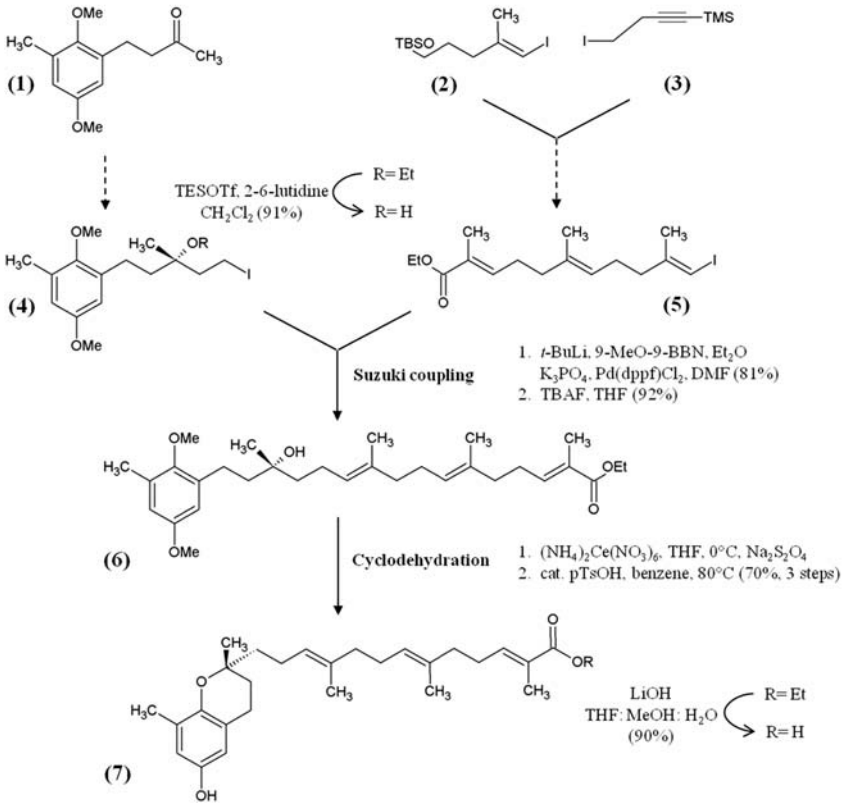

FIGURE 9.7 Stereo-controlled synthesis of garcinoic acid. *Adapted from D.J. Maloney, S.M. Hecht, Org. Lett. 7 (2005) 4297 4300.*

butan-2-one (**1**). The Negishi coupling of *tert*-butylsilyloxy-5-iodo-4-methylpent-4-ene (**2**) and 4-iodo-1-(trimethylsilyl)but-1-yne (**3**) yielded the vinyl iodide (**5**). Suzuki coupling of (**4**) and (**5**) gave the protected prenylated 1,4-benzoquinone, (2*E*,6*E*,10*E*,14*R*)-ethyl 14-hydroxy-16-(2,5-dimethoxy-3-methylphenyl)-2,6,10,14-tetramethylhexadeca-2,6,10-trienoate (**6**). The acid-catalyzed cyclodehydration followed by saponification leads to synthetic garcinoic acid (**7**) [34]. In principle, this synthesis route provides an alternative way to obtain the α- and δ-LCM, starting with synthetic garcinoic acid.

Semisynthesis of Long-Chain Metabolites From Garcinoic Acid

Garcinoic acid, either isolated from the various natural sources or chemically synthesized, can be used for the semisynthesis of α- and δ-LCM. Mazzini et al. reported the respective approach in 2009 [33]. A synthesis route leading to α-TOH was outlined, using the isolated compound from *G. kola* (obtained according to the procedure provided by Terashima et al. [25]) (Fig. 9.8).

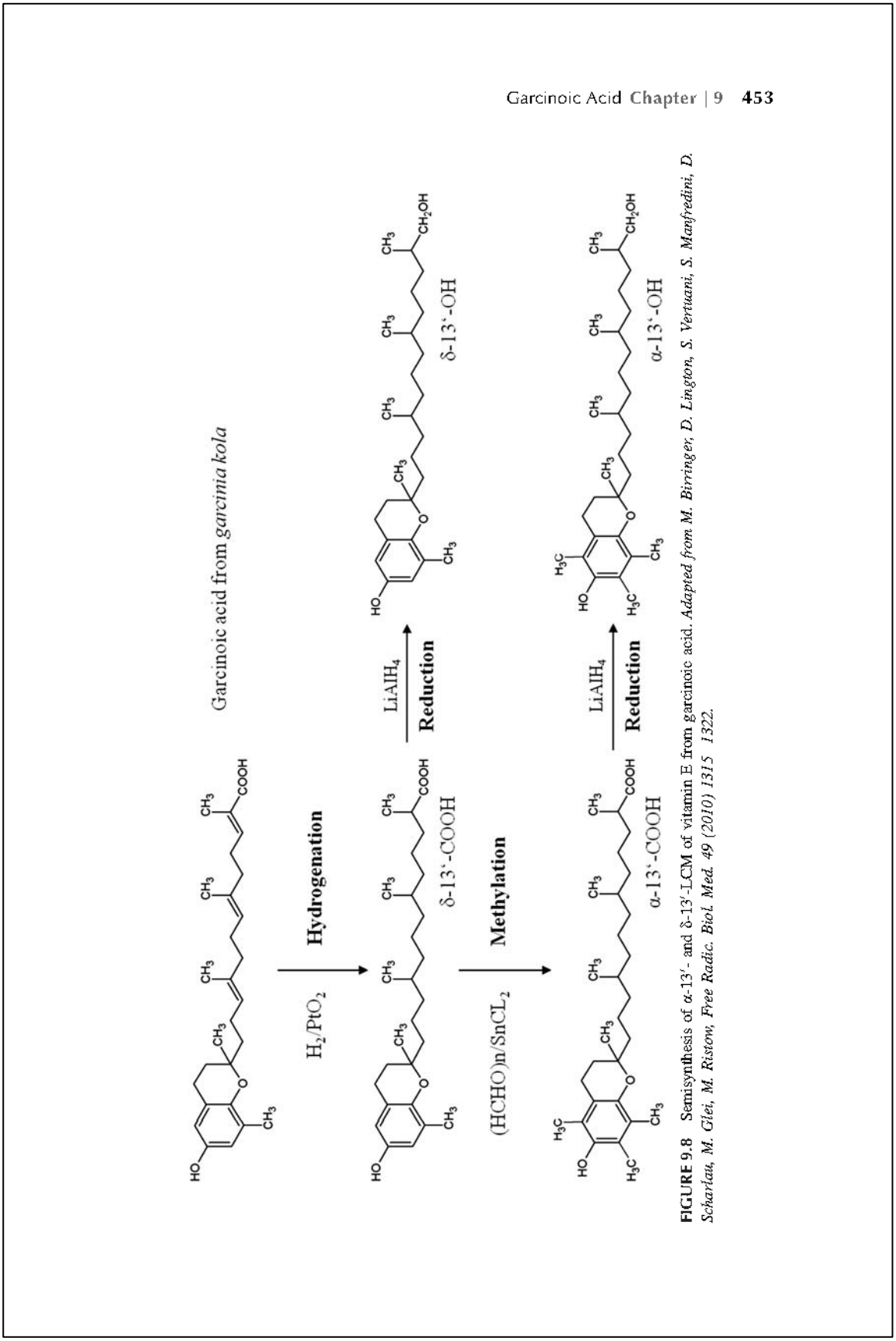

FIGURE 9.8 Semisynthesis of α-13'- and δ-13'-LCM of vitamin E from garcinoic acid. *Adapted from M. Birringer, D. Lington, S. Vertuani, S. Manfredini, D. Scharlau, M. Glei, M. Ristow, Free Radic. Biol. Med. 49 (2010) 1315–1322.*

Here, the unsaturated side chain of garcinoic acid is first hydrogenated in a platinum-catalyzed reaction to receive δ-13′-COOH. The corresponding α-LCM, α-13′-COOH, is obtained by permethylation of δ-13′-COOH, catalyzed by $SnCl_2$. A reduction with $LiAlH_4$ leads to α-13′-OH. To obtain α-TOH, the alcohol can be converted into a ditosylate derivative, and subsequently, the tosyl groups are removed by treatment with $LiAlH_4$ and heating in an aqueous basic solution (not shown). Finally, a hydroxy group resides at the chroman ring and the chain loses its functional moiety [33]. This synthesis route was reproduced by Birringer et al. later. Again, *G. kola*—derived garcinoic acid was used, but δ-13′-OH was derived by reduction of δ-13′-COOH with $LiAlH_4$, additionally. Hereby, the δ-LCM as well as the α-LCM can be obtained from garcinoic acid at sufficient purity for further usage in functional assays [87].

BIOACTIVITY OF GARCINOIC ACID, VITAMIN E, AND LONG-CHAIN METABOLITES

Several functions of vitamin E have been proposed until today. In the early days of vitamin E research, the focus was on the radical chain breaking and radical scavenging capacity of α-TOH, which is regarded as the most potent member of the vitamin E family in this respect [97]. However, Angelo Azzi was the first who provided evidence for further properties of α-TOH that are independent of its function as an antioxidant. He found that α-TOH regulates several cell functions via modulation of signal transduction, nuclear receptors, as well as gene and protein expression besides its function as a natural antioxidant [98,99]. T3 possess similar and sometimes even stronger biological activities than TOH; in particular, T3 show antioxidative, antiatherogenic, anticancer, antidiabetic, antiinflammatory, and neuroprotective properties [58,100]. Apart from the well-known functions of the different vitamin E forms, the bioactivity of their metabolites is not well understood.

Vitamin E metabolism has been studied intensively since the 1990s, but it took about a decade until the first groups were able to detect α-, γ-, and δ-13′-OH as well as the corresponding 13′-COOH metabolites in cell culture supernatants [76], in human liver cells [87], and also in human serum [101]. Current research on LCM is focused on their antiinflammatory properties. Investigations of different groups showed regulatory actions of the LCM on enzymes of the inflammatory cascade [102,103]. Further studies revealed antioxidative and cytotoxic effects [33,87], as well as regulatory properties in lipid metabolism [101]. Based on these studies, the LCM seem to have higher activity and modes of action different from those of the respective vitamin E forms. Garcinoic acid is a natural compound with high structural similarity to the LCM of δ-T3 (and identical to the 13-carbon side chain acid metabolite) [25], indicating that the bioactivity of this substance may be comparable to the LCM of TOH and T3. However, only a few studies on the biological actions of

garcinoic acid have been described so far. The acid exhibits high antioxidative potential [25,31] and antiproliferative effects [33]. However, almost nothing is known about its antiinflammatory or regulatory potential.

The following paragraphs provide an overview on the properties of garcinoic acid and the different LCM in comparison to their precursors.

Cytotoxicity

Recent animal studies on toxic effects of natural or nonnatural vitamin E forms and derivatives on reproduction and development revealed no toxic effects [104]. Physiological vitamin E intake can be increased up to 300 mg/day (mixture of TOH and T3, ~190 IU/day) without causing any complications [105,106]. No clear adverse effects have been described, even for short-term high-dose administration of vitamin E. However, persistent high-dose supplementation has been shown to interfere with blood clotting and is therewith associated to an increased risk of hemorrhagic stroke in animal studies [104]. In the past, TOH was considered to be a safe food additive [107], but an increase in total mortality after high-dose vitamin E intake was discussed during the last years [108]. However, excessive intake of vitamin E results in increased metabolite formation and excretion [109]. This could be a hint that the metabolites of vitamin E may cause noxious effects after a high-dose intake of vitamin E.

Cytotoxic Effects of Vitamin E

Reports on cytotoxic effects of vitamin E are inconsistent. There are considerable differences in the cytotoxicity of the different vitamin E forms. McCormick and coworkers investigated the cytotoxic potential of α-, γ-, and δ-TOH in RAW264.7 macrophages. Concentrations up to 60 μM γ-TOH and especially δ-TOH decreased cell viability by 50% and 90%, respectively, whereas α-TOH had no effect [110]. This has been confirmed in CEM/VLB100 and murine C6 glioma cells [111,112]. Experiments with δ-TOH in different cell types, such as MCF-7 cells, HepG2 cells, and fibroblasts, indicate that δ-TOH-triggered cytotoxicity may depend on the cell type. While δ-TOH incubation results in a massive reduction of viability in MCF-7 breast cancer cells and fibroblasts, no effect was observed for HepG2 liver cells [110]. The first hypothesis—the cell type—dependent cytotoxicity due to different intracellular accumulation of TOH—was disproved [110]. Another concept implies that the degree of methylation of the chroman ring is important for cytotoxicity [110].

In comparison to TOH, T3 show diverse cytotoxic effects. In A549 and U87MG cells, δ-T3 exhibited the highest cytotoxicity followed by γ- and α-T3. Further, the cytotoxicity of T3 derivatives also depends on the cell type [113]. Moreover, cell viability was also reduced in HepG2 liver cells by 40 μM of δ-T3 or γ-T3 [114]. Thus, T3 are able to reduce cell viability in cell types where TOH have no effect. Taken together, the δ-forms of TOH and T3 seem to be the most

cytotoxic vitamin E forms. Moreover, lower concentrations of T3 are needed compared to TOH. TOH and T3 are also known to affect cell proliferation. Antiproliferative effects of all TOH forms have been observed in C6 glioma cells with concentrations higher than 50 μM. Here, α-TOH and γ-TOH were the most potent proliferation inhibitors [112]. The underlying mechanism is probably a block of the cell cycle via p27-mediated inhibition of the cyclin E/cyclin-dependent kinase 2 complex [115] and by increased p53 expression [116]. In particular γ-TOH and δ-TOH, but not α-TOH, affect these pathways [112].

Similar effects can be induced by T3. Because of their higher reactivity, antiproliferative effects of T3 have been studied in cancer cells to use T3 as therapeutic reagents. T3—δ-T3 more effectively than γ-T3—reduced cell proliferation in HL-60, A549, and U87MG cells by induction of apoptosis [113,117]. Thus, TOH (cell cycle arrest) and T3 (apoptosis) exert their anti-proliferative effects via different mechanisms.

Metabolites of Vitamin E

As mentioned before, high doses of vitamin E increase formation of metabolites and their excretion. Therefore, TOH and T3 metabolites might contribute to cytotoxic effects of vitamin E. Studies of Conte et al. in 2004 provided first impressions of CEHC-mediated cytotoxic effects in cancer cell lines. In this work, γ-TOH, γ-T3, and γ-CEHC inhibition of cell proliferation were compared to their respective α-homologues. It should be emphasized that the γ-forms of TOH and T3 have higher transformation rates to CEHC than the respective α-forms. This has been evaluated in PC3, LNCaP, and HepG2 cells [118]. γ-T3 and γ-CEHC are the most potent inhibitors of cancer cell proliferation. At 10 μM, both compounds reduced proliferation of PC3 cells by 70−82%, while their α-analogues were less effective [119]. Francesco Galli and coworkers presume that this effect is triggered by a block of cyclin D1, but further investigations are needed to prove this concept [119]. In conclusion, the SCM are as effective as their precursors in inhibiting cell growth, with γ-forms being most potent.

In contrast to SCM, LCM are widely uncharted. Based on earlier results of Galli et al. and Conte et al. indicating that carboxy-SCM exhibit pro-apoptotic properties, Birringer et al. discovered similar effects for the 13′-LCM [87,118,119]. In this study, HepG2 cells were incubated with α-13′-COOH and δ-13′-COOH and α-13′-OH and δ-13′-OH. The carboxy metabolites appeared to be potent inducers of cell death, while the hydroxy metabolites did not affect cell survival. Furthermore, the δ-forms have been more active than the α-forms. This is reflected by the EC_{50} values of the two substances: 6.5 μM for δ-13′-COOH and 13.5 μM for α-13′-COOH [87], in comparison to α-TOH ($EC_{50} > 100$ μM) and α-CEHC, which showed very low antiproliferative effects at concentrations >10 μM [119]. This finding is in line with the observation that α-13′-COOH and δ-13′-COOH significantly increased the

ratio of apoptosis of HepG2 cells, compared to their metabolic precursors α-TOH and δ-TOH [87]. The treatment of HepG2 cells with α-13′-COOH and δ-13′-COOH also caused increased expression of caspase-3, which is a key enzyme of apoptosis. While δ-13′-OH slightly increased caspase-3 expression, α-13′-OH, α-TOH, and δ-TOH did not [87].

To sum up, the LCM show effects on cell proliferation and cell viability similar to those of their metabolic precursors, but there are significant differences in their activity and the LCM act at much lower concentrations.

Garcinoic Acid

Based on its structural similarities to δ-13′-COOH, it is hypothesized that garcinoic acid has comparable antiproliferative and cytotoxic properties as other vitamin E analogues. To confirm this hypothesis and to get more information about the structural requirements for antiproliferative properties, Mazzini et al. [33] investigated cell proliferation in glioma C6 cells after incubation with garcinoic acid. The acid reduced growth of C6 cells by 50% at concentrations of 10 μM. This effect has also been observed for α-CEHC and δ-CEHC in this study, indicating that the length of the side chain has barely influence on the antiproliferative properties [33,119]. Nevertheless, δ-13′-COOH and α-13′-OH showed higher inhibitory effects on proliferation of C6 cells than α- and δ-CEHC. This indicates that the presence of the carboxyl or hydroxyl group of the vitamin E metabolites enhances antiproliferative effects [33,87]. Based on the limited data on the cytotoxicity of garcinoic acid, its properties seem to be comparable to the other vitamin E metabolites. We found that garcinoic acid showed cytotoxic effects in the RAW264.7 mouse macrophage model system in which we revealed EC_{50} concentrations of about 5.5 μM (unpublished data).

The cytotoxicity of natural compounds is of particular interest for cancer treatment. Several plant-derived anticancer agents are already in clinical use. In particular, taxanes, camptothecines, vinca alkaloids, and podophyllotoxins are worth mentioning [120]. The compounds exert different modes of action, but all have been shown to have antiproliferative effects on cancer cells [121–124]. This is also a characteristic of garcinoic acid, making it interesting for cancer research. Although the effects of garcinoic acid on cancer cells and the underlying mechanisms have still to be characterized, one promising property is already known: garcinoic acid inhibits DNA polymerase β with an IC_{50} of about 4 μM [34]. Compared to other natural DNA polymerase β inhibitors, garcinoic acid is one of the most potent ones (reviewed in Ref. [125]). Cells deficient in DNA polymerase β activity are hypersensitive to certain chemotherapeutic agents due to their impaired ability to repair induced DNA damage [126]. For this reason, the further characterization of the cytotoxic effects of garcinoic acid is of great interest. If garcinoic acid is able to induce DNA damage and simultaneously to suppress DNA damage repair mechanisms, it might be a

powerful agent for cancer treatment. However, the effects of garcinoic acid should first be well characterized in cellular systems before experiments in animal models or even clinical trials in humans can be conducted.

Antioxidative Properties

The antioxidative properties of the different vitamin E forms and metabolites have been extensively studied during the last decades, considering α-TOH as the most important antioxidant, mainly due to the protection against peroxidation of polyunsaturated fatty acids (PUFA[8]) in phospholipids of cellular membranes and plasma lipoproteins, a finding made at least in vitro [56,127]. Higher PUFA intake requires higher vitamin E supply to provide adequate antioxidative protection against lipid peroxidation. Unsaturated fatty acids tend to form radicals, which can be scavenged by the free hydroxyl group at the chroman ring of α-TOH; the reaction product is afterward excreted to bile as α-TOH hydroquinone [128]. All TOH and T3 forms exhibit antioxidative properties. Besides the free hydroxyl group, the mobility of the molecule in cellular membranes is a crucial factor [97,129]. The T3 have higher membrane mobility due to their unsaturated side chain. This should lead to an increase in their antioxidative capacity compared to the respective TOH forms. Yoshida and coworkers compared the effects of either TOH or T3 treatment on peroxyl radical scavenging, but no differences were detectable in membrane uptake or reactivity. However, another investigation on leptosome complexes revealed different results. In this experiment, α-T3 and α-TOH were integrated separately into synthetic membranes. Afterward, lipid peroxidation was induced in another part of the liposomal complex. It appeared that α-T3 was more potent in inhibiting peroxyl radical formation than its TOH equivalent. The more pronounced antioxidative potential of α-T3 seemed to be a result of its better intermembrane mobility, making α-T3 able to reach the radicals faster than α-TOH [130]. This observation has been confirmed by Serbinova and coworkers in rat liver microsomes [97]. However, there are also studies showing similar antioxidant activities of TOH and T3 [130,131].

In addition to membrane mobility, the number of methyl groups of the chroman ring increases the antioxidative capacity of TOH and T3. Despite this, the position of the methyl group in relation to the hydroxyl group at the chroman ring is important. For this reason, the α-forms have higher antioxidative potential than β-, γ-, and δ-derivatives. This has been shown for TOH and T3 in liposomal membranes. After induction of peroxyl radical−triggered lipid peroxidation, the α-derivatives were the most potent inhibitors of oxidative stress. The antioxidant activity decreased from α

8. Polyunsaturated fatty acids are a class of fatty acids characterized by more than one double bond; they are often essential for human nutrition.

through β to γ down to δ [130]. A further investigation in rat serum confirmed this observation [132]. Apart from these results, there are several in vitro studies indicating a reverse order of antioxidant efficiency with α-TOH being the least potent compound compared to δ- and γ-TOH [133,134]. In conclusion, TOH and T3 are highly potent antioxidants with a theoretically decreasing antioxidant activity from α- through β- to γ- and down to δ-forms. Furthermore, T3 seem to be more active than the respective TOH equivalents.

Due to the similarity of the chemical structure of garcinoic acid with T3, comparable antioxidant activities of these compounds can be expected. The antioxidative properties of this natural compound have been investigated in two independent studies. Okoko and coworkers used a methanolic extract from *G. kola* seeds for in vitro experiments. First, the extract was divided into five fractions by TLC. Afterward, the radical scavenging abilities of each fraction were compared to those of vitamin C. The fraction with the highest activity in hydroxyl radical scavenging was further investigated via HPLC analysis. Chromatographic fractioning and spectroscopic analysis revealed four compounds, including *Garcinia* biflavones GB1 and GB2, garcinal, and garcinoic acid [31]. The combination of these four compounds had a 40% higher antioxidative activity than vitamin C at a concentration of 0.5 μg/mL. Further investigations in U937 macrophage cells revealed inhibitory effects on nitric oxide formation [31]. However, Okoko and coworkers were not able to draw a conclusion whether a single compound or the combination of the four substances is responsible for the observed effects. The lack of compound-specific investigations is a crucial limitation of this study. In another investigation, the antioxidative potential of garcinoic acid has been compared to α-TOH using antioxidant activity assays. Terashima et al. found that the antioxidant activity of the natural product was 1.53 times that of α-TOH. This value was comparable to δ-T3 (1.47) and δ-TOH (1.53), molecules sharing high structural similarity to garcinoic acid [25]. Terashima and coworkers chemically modified garcinoic acid by shortening of the side chain. It appeared that the antioxidative activity was significantly affected by structural features, i.e., the shorter the side chain the higher the antioxidative potential. The garcinoic acid analogue with the shortest side chain had 18.7 times higher antioxidant activity than α-TOH [25]. To conclude, garcinoic acid seems to be one of the most potent antioxidative compounds in *G. kola* seeds with an antioxidant activity comparable to compounds such as δ-TOH and δ-T3.

The lack of in vivo studies with garcinoic acid makes predictions difficult whether the antioxidative capacity of garcinoic acid can contribute to drug development and disease treatment. Natural antioxidants in general are believed to have beneficial effects on different diseases. One of the best investigated groups of natural antioxidants are the polyphenols. Compounds such as quercetin, resveratrol, and curcumin are well-investigated members of this class of compounds that have almost similar antioxidative properties as garcinoic acid. All three substances are potent radical scavengers, especially

for hydroxyl radicals [135–137]. Furthermore, quercetin and curcumin have inhibitory effects on nitric oxide formation in different cell types [138,139]. In contrast to garcinoic acid, the use of these polyphenolic compounds for the treatment of diseases in which oxidative stress is involved has already been investigated in mouse models and humans. For example, natural antioxidants showed beneficial effects in nonalcoholic fatty liver disease (NAFLD[9]) and Alzheimer disease (reviewed in Refs. [140,141]). NAFLD is a metabolic disorder associated with high levels of free fatty acids and an increased cardiovascular and liver-related morbidity [142]. High oxidative and inflammatory damage in hepatocytes can also lead to nonalcoholic steatohepatitis (NASH[10]) [143]. Experiments in mice fed a Western diet showed that quercetin lowers oxidative stress in hepatocytes, which in turn leads to reduced liver steatosis [144]. In addition, resveratrol showed promising effects for NAFLD patients in a controlled clinical trial, mainly through lowering inflammatory markers and the reduction of oxidative stress [145]. Resveratrol was further used in studies on Alzheimer disease. Studies demonstrated the importance of neuroinflammation and oxidative stress in the pathogenesis of this disease. One of the most important factors contributing to the development of Alzheimer disease is β-amyloid, because of its ability to generate superoxide anions and α-carbon-centered radicals. The high ROS production caused by β-amyloid may lead to neuronal death [146,147]. Due to its antioxidant activity, resveratrol was used for the treatment of Alzheimer disease in rats, where the compound protected glioma cells from β-amyloid-triggered oxidative damage [148]. Furthermore, curcumin also protected neuronlike PC12 cells from β-amyloid toxicity and displayed neuroprotective effects larger than those of well-known antioxidants such as α-TOH [149]. Besides studies in cellular models, Lim and coworkers have also shown that dietary curcumin suppresses inflammation and oxidative damage in the brain of Tg2576 mice [150]. Furthermore, the epidemiological study by Ganguli and coworkers provides evidence that the Indian population, known for its curcumin-rich diet, shows reduced prevalence of Alzheimer disease compared to the US population [151].

Based on the fact that oxidative stress is a crucial factor for the development of both diseases and natural antioxidants have already shown promising effects on disease prevention, it can be hypothesized that the antioxidative properties of garcinoic acid bear potential for its use in drug development as well as disease prevention and treatment. The well-known effects of other

9. Nonalcoholic fatty liver disease is characterized by the accumulation of fat in the liver of people with no or low alcohol consumption that can lead to inflammation and scarring of the liver.
10. Nonalcoholic steatohepatitis is hallmarked by the accumulation of fat in the liver of people with no or low alcohol consumption accompanied by chronic inflammation, progressive scarring, and cirrhosis of the liver.

natural antioxidants in the prevention of NASH and Alzheimer disease are a promising starting point for in vivo experiments with garcinoic acid.

Apart from the vitamin E isoforms and garcinoic acid, almost nothing is known about the antioxidant activity of the 13′-LCM. Because of their high reactivity, the two LCM 13′-OH and 13′-COOH may act as prooxidants. To prove this hypothesis, Birringer et al. [87] investigated 13′-LCM-triggered ROS production. HepG2 cells were treated with α-13′-OH, δ-13′-OH, α-13′-COOH, and δ-13′-COOH. The corresponding TOH forms were used as controls. Generation of intracellular and mitochondrial ROS was measured via dichlorofluorescein assay [152]. Incubation with 10 μM α-13′-COOH or δ-13′-COOH increased intracellular ROS formation while α-13′-OH, δ-13′-OH, and both TOH forms showed no effect. Similar effects have been observed for mitochondrial ROS production. Here, α- and δ-13′-COOH increased mitochondrial ROS production by 30–50% while the other compounds had no effect. A decrease in mitochondrial ROS production was observed only for δ-TOH [87]. In conclusion, α-13′-COOH and δ-13′-COOH seem to have strong prooxidant potential while α-13′-OH and δ-13′-OH do not act as prooxidants. Due to the structural similarity to the α-13′-COOH and δ-13′-COOH, it can be expected that garcinoic acid exhibits a similar prooxidant potential, but this has to be confirmed experimentally. These observations differ from the results for the antioxidant effects of the different TOH and T3 forms. Particular attention should be paid to studies showing that α-TOH can possibly act as prooxidant [153,154].

Antiinflammatory Actions

Multiple cell types of the innate immune system and paracrine-acting as well as autocrine-acting mediators contribute to the complex process of inflammation. Here, the interplay of proinflammatory and antiinflammatory mediators is vital for the outcome of the inflammatory process, i.e., resolution or chronic inflammation. CVD and cancer, two of the leading causes of death worldwide, are inflammatory diseases, thus highlighting the importance of research for new antiinflammatory treatment approaches. Moreover, diseases of civilization, such as diabetes and obesity as well as asthma, rheumatoid arthritis, osteoporosis etc., have been linked to inflammation.

For this reason, the natural modulators of inflammation are of particular interest. Although several mediators of inflammation and underlying pathways have been identified, we here draw attention to the factors only, which have been investigated in the context of LCM and garcinoic acid.

Cyclooxygenases and Their Lipid Mediator Products

Eicosanoids comprise a group of lipid mediators involved in inflammation, which include prostaglandins, thromboxanes, leukotrienes (LT) and lipoxins. All eicosanoids are metabolically derived from arachidonic acid. Key enzymes

of the conversion of arachidonic acid to eicosanoids are COX1 and COX2 as well as lipoxygenases (LOX; the reader is referred to the section "Vitamin E and Lipoxygenases"). Arachidonic acid is released by action of phospholipases A_2 from phospholipids of the cell membrane. The bifunctional COX (cyclooxygenation and peroxidation function) forms prostaglandin G_2 from arachidonic acid by cyclization and addition of two molecules of oxygen and reduces it further to prostaglandin H_2. This endoperoxide serves as substrate for specific synthases and isomerases, which form prostaglandins of the E_2, F_2, D_2, and I_2 series as well as thromboxane A_2 [155].

While COX1 is constitutively expressed, COX2 can be induced by a variety of proinflammatory stimuli. Hence, COX2 is regarded as the more important source of eicosanoids during inflammation. All of the abovementioned prostaglandins are implicated in proinflammatory actions (reviewed in Ref. [156]).

Vitamin E Modulates Prostaglandin E_2 Release and Cyclooxygenase Activity

Tocopherol Inhibit Cyclooxygenase Activity

The release of prostaglandin E_2 (PGE_2) is widely used as a marker for the activity of COX. The effect of TOH on the release of PGE_2 has been studied in several cell types and settings. In BV-2 microglia cells the induction of PGE_2 by LPS could be attenuated by α-TOH dose-dependently. While 25 μM showed no effect, 50 μM diminished the effect significantly and 100 μM almost completely blocked the induction [157]. An interesting finding was made in human aortal endothelial cells: α-TOH induced the release of PGE_2 dose-dependently in concentrations above 10 μM. In contrast, COX activity, measured as conversion of exogenous arachidonic acid to PGE_2, was attenuated by α-TOH at 10 μM or higher. The authors postulated that α-TOH induces (1) the release of arachidonic acid from membrane phospholipids and (2) the expression of $cPLA_2$. The discrepancy in the abovementioned results is explained by a more relevant effect of α-TOH on substrate release (i.e., the release of arachidonic acid from membrane phospholipids) than on COX activity [158].

These findings implicate that the effects of TOH on PGE_2 release depend on the cell type. However, similar findings were made in macrophages. In peritoneal macrophages obtained from rats treated with 5 mg/day α-TOH (i.p.) for 6 days, the production of PGE_2 in response to different stimuli was diminished. Interestingly, macrophages from control animals showed a response similar to untreated control cells, when preincubated with α-TOH [159]. In a different approach with peritoneal macrophages the most effective reduction of PGE_2 production was observed with δ-TOH (1.25−12.5 μM) and α-TOH (12.5−150 μM). γ-TOH was less effective and β-TOH had no effect (up to 12.5 μM). Interestingly, all TOH forms reduced COX activity, measured

as the conversion of PGE_2 from exogenous arachidonic acid. Again, δ-TOH was most potent followed by β-, α-, and γ-TOH in descending order [160]. Thus, the substitution of the chroman ring seems to be important for the modulation of PGE_2 synthesis. However, it is possible that the different TOH forms act in different ways, either on substrate availability or on COX activity.

Are Tocotrienols the More Potent Vitamin E Form?

T3 have also been shown to be potent inhibitors of PGE_2 release. In malign mammary epithelial cells, PGE_2 release was reduced about 50% of controls by 3 μM γ-T3 [161]. Different effects were observed in mouse RAW264.7 macrophages stimulated with LPS to induce PGE_2 release and subsequently incubated with three different T3 forms at 10 μg/mL. While γ-T3 showed no effect, δ-T3 was the most potent inhibitor with about 55% reduction followed by a T3-rich fraction and α-T3. Surprisingly, α-TOH increased the effect of LPS induction [162]. In IL-1β-stimulated A549 lung epithelial cells, γ-T3 was as effective as δ-TOH in inhibiting release of PGE_2. The IC_{50} for both compounds were about 1−3 μM. γ-T3 was more potent than its γ-TOH counterpart (IC_{50} of 6−7 μM), while α-T3 exerted only weak inhibitory action (20% at 20 μM), and α- and β-TOH were completely ineffective below 50 μM [84]. The aforementioned results suggest that the T3 are more potent inhibitors of COX activity than their respective TOH forms. However, the substitution pattern of the chroman ring appears to be also a major determinant for the effectivity of the compound.

Tocopherol Metabolites Outclass Their Metabolic Precursors

While little is known about the bioactivity of TOH LCM in general, some studies focused on their effects on COX activity. We recently reported that α-13′-COOH is a potent COX-regulating metabolite. In mouse RAW264.7 macrophages, the upregulation of COX2 mRNA and protein by LPS and the subsequent increase in PGE_2 release was diminished by α-13′-COOH and α-TOH. Whereas α-TOH reduced PGE_2 production about 55%, α-13′-COOH abolished PGE_2 production almost completely. These findings are of particular significance as 100 μM α-TOH was less effective than 5 μM of α-13′-COOH. This underlines the higher effectivity of the LCM. In addition to PGE_2, the LPS-induced formation of further arachidonic acid−derived eicosanoids, namely prostaglandin D_2 and prostaglandin $F_{2\alpha}$, was blocked by α-13′-COOH. In contrast, α-TOH did not diminish the induction by LPS significantly [102]. In agreement with this, Jiang et al. reported no effect of 50 μM α-TOH on PGE_2 production in lung epithelial cells [84]. Compared to α-TOH, δ-TOH is more potent in inhibiting PGE_2 production (vide supra). In contrast to Wallert et al., Jiang et al. used no synthetic LCM, but cell culture medium collected from cells treated with TOH, containing the self-synthesized metabolites 9′-COOH, 11′-COOH, and 13′-COOH. An intact-cell assay with preinduced

COX and arachidonic acid as substrate revealed that the medium containing the δ-metabolites is superior to that with γ-metabolites in inhibiting COX activity. Unfortunately, the authors used a cell line that is unable to metabolize α-TOH to its respective carboxychromanols [85,86], resulting in no effect of α-TOH in this assay. δ-9′-COOH and δ-13′-COOH isolated from cell culture supernatants inhibited COX2 with IC_{50} of 6 or 4 μM, respectively. However δ-9′-COOH was unable to inhibit activity of purified COX1 and COX2 enzymes in concentrations <20 μM. In contrast, δ-13′-COOH was highly potent with an apparent IC_{50} of 5 μM for COX1 and 4 μM for COX2, which is comparable to ibuprofen. Only weak inhibition of both COX isoforms was shown for the SCM α-CMBHC (α-5′-COOH; $IC_{50} > 140$ μM) and γ-CEHC (γ-3′-COOH; $IC_{50} > 300$ μM) [84]. This finding indicates that LCM rather than SCM may be responsible for the antiinflammatory effects of TOH. This assumption is supported by the fact that A549 lung epithelial cells are not able to produce SCM [85,86]. Anyway, δ-SCM would be preferable for comparison, as the structure of the chroman ring likely influences the effectivity.

Garcinoic Acid: A New Player on the Court?

To date, no systematic investigation of the modulation of COX activity by garcinoic acid, the principal δ-13′-LCM of δ-T3, has been published. With respect to its structural similarities, garcinoic acid shares the chroman ring with δ-TOH, which has been shown to be the most potent TOH in this context [160]. In addition, δ-T3 is more effective in modulating COX activity than the other T3, which in turn can be considered more effective than TOH [84,162]. The unsaturated chain is a structural feature of garcinoic acid shared with T3. For this reason, we expect that garcinoic acid is more potent in modulating COX than TOH. As garcinoic acid carries a carboxylic acid moiety, one can compare it to the 13′-carboxychromanols generated from TOH. In particular, 13′-COOH have been shown to be substantially more effective in inhibiting COX activity than their metabolic precursors [84,102]. Based on these observations, garcinoic acid is likely more potent than TOH and comparable to (δ-)T3 or its LCM, respectively. However, experiments are required to confirm whether this hypothesis holds true.

Vitamin E and Cyclooxygenase Expression

While the effects of the different vitamin E forms on COX activity are evident, the underlying mechanisms are not yet fully resolved. A common way to decrease the activity of an enzyme—in addition to its inhibition—is its downregulation. As COX1 is constitutively expressed, no regulation is expected nor has been shown experimentally [158,161,162]. Divergent results have been obtained with respect to the influence of vitamin E on COX2 expression. In murine microglia cells, 50 μM α-TOH abolished LPS-induced gene expression and 100 μM moreover reduced protein synthesis of COX2,

likely via NFκB [157]. However, contradictory results were obtained in other studies. COX2 levels were reduced neither by 100 μM α-TOH in LPS-treated murine macrophages [102] nor by 60 μM α-TOH in IL-1β-stimulated human lung epithelial cells [158]. For the suggested molecular mode of action on COX activity, the reader is referred to the section "Tocopherol Inhibit Cyclooxygenase Activity."

In contrast to TOH that may exert posttranscriptional effects on COX activity, T3 have been shown to downregulate COX expression. In LPS-treated RAW264.7 macrophages, 10 μM of α-, γ-, and δ-T3 blocked COX2 expression while α-TOH did not [162]. In line with this, 10 μM of γ-T3 downregulated constitutive COX2 expression in human pancreatic cancer cells and 50 μM completely blocked the expression [163]. These findings are supported by further studies, characterizing γ-T3 [161,164] and δ-T3 [165] as highly efficient suppressors of COX2 expression. Interestingly, in both studies comparing the effects of T3 forms on COX2 expression, δ-T3 was the most potent one [162,165]. The higher ability of δ-T3 to diminish COX2 activity is in accordance with results for the different TOH forms. However, the ability to regulate COX2 expression seems to be a characteristic of T3.

Effect of Long-Chain Metabolites of Vitamin E on Cyclooxygenase 2 Expression

Based on the findings for TOH and T3, it can be assumed that α-TOH LCM are rather ineffective in regulating expression of COX2. Surprisingly, Wallert et al. reported significant blocking of LPS-induced expression of COX2 by α-13′-COOH in murine RAW264.7 macrophages: preincubation with 5 μM α-13′-COOH and subsequent coincubation with LPS significantly diminished the effect of LPS on COX2 expression at mRNA and protein levels. In contrast, 100 μM of α-TOH showed no significant effect [102]. These results show that the α-LCM act in a different fashion and at lower concentrations than their respective metabolic precursors. The underlying pathways have not been elucidated so far and remain to be investigated.

Effect of Garcinoic Acid on Cyclooxygenase 2 Expression

So far, no studies have been published that investigate the effects of garcinoic acid on COX2 expression. Due to the unsaturated chain, garcinoic acid is structurally comparable to δ-T3 but also shares similarities with α-13′-COOH. Considering this, it can be assumed that garcinoic acid may also interfere with the LPS-mediated upregulation of COX2. Preliminary results of our group indicate that garcinoic acid indeed has the potential to block the LPS-induced upregulation of COX2 mRNA as well as protein (unpublished data). However, this is merely a first hint and further experiments are needed. Nevertheless, garcinoic acid would not be the first compound isolated from plants for the treatment of inflammatory diseases in folk medicine. Well-known examples

are curcumin from *Curcuma longa*, capsaicin from *Capsicum* species, and epigallocatechin-3-gallate from *Camellia sinensis* [166]. All of these compounds have been shown to inhibit COX2 expression [167,168]. Especially *C. longa* has been used for centuries in Ayurvedic medicine to treat inter alia the inflammation-related diseases asthma, rheumatism, and diabetes [169]. Today, more than 50 completed clinical trials with curcumin display the interest in this valuable ingredient of *C. longa* in modern medicine. As with *C. longa*, *G. kola* is used in folk medicine to treat inflammation-related diseases (the reader is referred to the section "*Garcinia kola*"). Despite kolaviron, garcinoic acid has now been identified as an antiinflammatory active ingredient of *G. kola*. In principle, garcinoic acid is an interesting natural compound with antiinflammatory actions that should be further characterized. Possibly, the properties of kolaviron and garcinoic acid can be used jointly in the form of a *G. kola* nut extract to treat CVD, cancer, and other diseases of civilization.

Vitamin E and Lipoxygenases

Lipoxygenases and Their Lipid Mediators

LT are formed by LOX, a family of enzymes with four subclasses, namely 5-, 8-, 12-, and 15-LOX, which are classified according to the position at which these enzymes catalyze the dioxygenation of PUFA. The release of arachidonic from membrane phospholipids by $cPLA_2$ is crucial for LT synthesis. 5-LOX catalyzes the oxidation of arachidonic acid and thus the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which in turn is converted to LTA_4 by the same enzyme. LT A_4 is the precursor of LTB_4 or LTC_4, which are in turn the precursors of LTD_4 and LTE_4, respectively, formed by LTA_4 hydrolase and LTC_4 synthase, respectively [170].

Tocopherols Inhibit Lipoxygenase Activity

The first demonstration of 5-LOX inhibition by TOH was published in 1985 [171]. It was shown that α- and γ-TOH inhibit the conversion of arachidonic acid to 5-HPETE by 5-LOX from potato tubers. Interestingly, the inhibition was as efficient as with known 5-LOX inhibitors, such as nordihydroguaiaretic acid and butylated hydroxytoluene, and furthermore irreversible and noncompetitive with arachidonic acid [171]. LT B_4 is a major product of the 5-LOX pathway (vide supra) and is thus widely analyzed in activity and signaling studies. In 1999, Devaraj and Jialal noticed that preincubation of human peripheral blood mononuclear cells (PBMC) with α-TOH (but not β-TOH) impaired the release of IL-1β in response to LPS. Treatment of the cells with LTB_4 restored IL-1β release. By the use of 5-LOX inhibitors it was confirmed that 5-LOX mediates the effects of α-TOH. Furthermore, α-TOH diminished LTB_4 release [172]. A later study of the same group confirmed these results. PBMC isolated from α-TOH-supplemented healthy subjects showed impaired ability to produce TNFα in response to LPS compared to

cells obtained at baseline or after washout. Preincubation of LPS-stimulated PBMC with 50 or 100 μM α-TOH as well as 5-LOX inhibitors showed the same effect. The impaired TNFα release could be restored by LTB_4 [173]. In vitro experiments show that concentrations of 25 μM of α-TOH are not sufficient to inhibit release of TNFα. This raises the question whether a supplementation with 1200 IU/day (corresponds to about 800 mg/day) is sufficient to achieve the required plasma levels of α-TOH. It might be possible that the α-LCM mediate or contribute to the effects observed in vivo, but this remains to be shown experimentally.

Two human trials in hemodialysis patients support the abovementioned findings [174,175]. Patients under hemodialysis exhibit increased 5-LOX levels in their PBMC. In these studies, patients were subjected to α-TOH administration to improve oxidative stress markers [174]. Supplementation with α-TOH, 300 mg/day i.m., 600 mg/day orally [150], or via vitamin E-coated cuprammonium rayon membranes [151] for 4 weeks diminished LTB_4 release and 5-LOX activity. The expression of 5-LOX was not affected by the treatments [175,176].

Although there is evidence that TOH are capable of inhibiting 5-LOX activity and LTB_4 production, further research is required. The majority of studies were done on α-TOH, but the different vitamin E forms seem to act differently and there might be more potent forms [171–173]. Furthermore, α-TOH was administered in the mentioned human trials and the effects were attributed to the TOH itself, regardless of metabolic conversions.

Effects of Metabolites and Tocotrienol on Lipoxygenase Activity

Despite the observation that T3 inhibit 12-LOX activity (reviewed in Ref. [177]), little is known about T3 and their effects on LOX. In fact, just a single study addressed effects of T3 on 5-LOX. In this study, γ-T3 was compared to different TOH forms and δ-13′-COOH [103]. For this, HL60 cells were differentiated into neutrophils and eosinophils to induce 5-LOX expression. Activity of 5-LOX was subsequently stimulated by different concentrations of the calcium ionophore A23187 and measured as formation of LTB_4 and LTC_4. In cells incubated with 1 μM, α-TOH was less effective (IC_{50} = 60 and 40 μM, respectively) than its γ- and δ-counterparts (IC_{50} = 5 μM). Interestingly, γ-T3 was as effective as γ-TOH, and δ-13′-COOH was the most potent compound tested (IC_{50} = 4 μM). Strikingly, δ-13′-COOH inhibited formation of LTB_4 with an apparent IC_{50} of 7 μM, when cells were stimulated with 5 μM A23187, while none of the other vitamin E forms was able to inhibit 5-LOX activity in this setting with concentrations up to 50 μM. The superiority of δ-13′-COOH was confirmed in a cell-free assay with recombinant 5-LOX. Here, the LCM efficiently inhibited the activity of 5-LOX with an IC_{50} of 0.5–1 μM, while all the other vitamin E forms failed to inhibit 5-LOX with concentrations of up to 50 μM. The efficiency of the carboxychromanol is thus similar to that of zileuton, a specific inhibitor of the 5-LOX-activating protein. For this

reason, δ-13′-COOH is thought to inhibit 5-LOX directly. However, final evidence is pending. An alternative way by which δ-13′-COOH may modulate 5-LOX activity is by inhibiting the increase in intracellular Ca^{2+} levels in response to N-formylmethionine leucyl-phenylalanine or thapsigargin. In this respect, the metabolite was superior to its metabolic precursor δ-TOH, which failed to inhibit the induction in calcium influx [103].

Garcinoic Acid and Lipoxygenase Activity

No experimental data regarding garcinoic acid and LOX activity are currently available, but based on the observation that the structurally related LCM δ-13′-COOH is a potent inhibitor of 5-LOX activity, garcinoic acid may likely exert similar effects on this enzyme. The finding that γ-T3 is also able to inhibit 5-LOX activity with an efficiency comparable to γ-TOH further supports this hypothesis, because the unsaturated chain has obviously no effect on the inhibitory capacity. The same may likely be true for garcinoic acid, but this has to be confirmed experimentally. 5-LOX and its products are involved in many inflammation-related diseases, including CVD, cancer, osteoporosis, inflammatory bowel disease, rheumatoid arthritis, skin diseases, and bronchial asthma. The latter is the major 5-LOX-associated disease and zileuton, the only approved 5-LOX inhibitor so far, is available for treatment [178]. Nevertheless, zileuton has two major drawbacks, liver toxicity and a short half-life [179]. There is thus an urgent need to find new potent 5-LOX inhibitors. Many natural products have been identified as 5-LOX inhibitors (reviewed in Ref. [178]). However, most of them are not well characterized and far from use as drugs [178]. Flavocoxid, a mixture of the bioflavonoids baicalin from *Scutellaria baicalensis* and catechins from *Acacia catechu*, made it to a phase III trial but the problem with this natural 5-LOX inhibitor is the reported risk of acute liver injury [180]. Garcinoic acid could line up with the known natural 5-LOX inhibitors, with the potential advantage of modification of multiple inflammatory pathways simultaneously (the reader is referred to the respective chapters on COX). Furthermore, extracts of *G. kola* have hepatoprotective effects [23], so a nut extract might exert effects on 5-LOX without liver injury. Moreover, garcinoic acid could be hepatoprotective itself, as the related structures TOH and T3 have been reported to be beneficial for liver health repeatedly [181,182]. If garcinoic acid is indeed a 5-LOX inhibitor, its exact mechanism of action should be investigated to assess its clinical potential. The lack of 5-LOX inhibitors with satisfying properties shows the need of new sources for their development and garcinoic acid is a promising candidate.

Modulation of Lipid Homeostasis

In addition to inflammation, dysbalanced lipid homeostasis is a key factor for diseases such as atherosclerosis. A plethora of signaling pathways and cellular

processes are required to regulate lipid homeostasis, involving uptake, intracellular trafficking and storage, metabolism, as well as efflux of lipids. The following sections will only focus on that parts of lipid metabolism that have been linked to the LCM so far, namely, expression of the scavenger receptor cluster of differentiation 36 (CD36), uptake of oxidized LDL (oxLDL), and phagocytosis as well as intracellular lipid storage. These are essential elements of macrophage foam cell formation, which in turn is a key event in the pathogenesis of atherosclerosis.

Tocopherols and Macrophage Foam Cell Formation

Macrophage-derived foam cells contribute significantly to the pathogenesis of atherosclerosis. This cell type is therefore studied extensively with respect to its role in inflammation and lipid metabolism. CD36 is a scavenger receptor that significantly contributes to the uptake of oxLDL and is thus involved in the accumulation of cholesterol in intracellular lipid droplets, a hallmark of macrophage foam cells. Therefore, factors that modulate CD36 expression and the uptake of oxLDL are of particular interest. The ability of TOH to modulate the regulation of CD36 and the uptake of oxLDL as well as subsequent processes has been described in several studies: α-TOH is able to suppress the upregulation of CD36 during macrophage differentiation [183,184]. Furthermore, it blocks the upregulation of CD36 in response to oxLDL in THP-1[11] macrophages [185] and to modified LDL in PBMC-derived macrophages [183]. Moreover, the uptake of oxLDL can be decreased by α-TOH in several macrophage models [183–185]. The incubation with oxLDL causes a lipid accumulation in macrophages, which can be also prevented by α-TOH [185]. In line with this, the accumulation of cholesteryl esters in response to modified LDL is diminished in α-TOH-treated macrophages [183].

The regulatory effects of α-TOH on CD36 have been observed also in mice. Apolipoprotein E-knockout mice fed a diet supplemented with 100 mg/kg α-TOH per day for 8 weeks showed a reduced extent of atherosclerotic lesions as well as the expression of CD36 therein and serum concentrations of oxLDL than the respective control group [186]. Similar findings were obtained in LDL-receptor-knockout mice. Here, supplementation with α-TOH acetate and α-TOH (equivalent to 50 IU vitamin E per kilogram of diet, ad libitum) for 18 months resulted in a decrease in lesional and nonlesional expression of CD36 [187].

These findings are also supported by results from liver disease research. The HepG2 liver cell line shows decreased CD36 expression when treated with α-TOH [188]. Rats fed a diet enriched with 80 IU/kg diet (ad libitum) α-TOH

11. THP-1 cells are a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 monocytes can be differentiated into macrophages using phorbol 12-myristate 13-acetate.

acetate showed reduced hepatic CD36 mRNA levels compared to controls [189], and a comparable result was obtained with merely 6 mg/kg of the diet α-TOH combined with 11 mg/kg of the diet γ-TOH ad libitum [190]. A study with guinea pigs points to a posttranslational regulatory mechanism of α-TOH decreasing CD36 protein levels in the liver [191].

Effects of Long-Chain Metabolites and Garcinoic Acid on Macrophage Foam Cell Formation

A surprising result was obtained when we examined the effects of LCM on CD36 expression. In contrast to the downregulatory potential of 100 μM α-TOH, its LCM α-13′-OH and α-13′-COOH upregulated CD36 mRNA and protein in human THP-1 macrophages and human PBMC-derived macrophages obtained from healthy volunteers with as little as 10 and 5 μM, respectively. Generally, primary cells showed a slightly lower response. In addition, the increase in CD36 expression by oxLDL was attenuated by α-TOH and markedly augmented with the LCM [101].

Given the LCM-induced CD36 expression, an increase in oxLDL uptake is expected, but this was not the case. Treatment with LCM before addition of oxLDL led to decreased oxLDL uptake in THP-1 and PBMC-derived macrophages. In line with this, the accumulation of neutral lipids by oxLDL was attenuated in LCM-pretreated cells [101]. We found that garcinoic acid also induces the expression of CD36 in the nonproliferating THP-1 macrophage model. Here, the effectivity of garcinoic acid was comparable to that of the α- and δ-LCM (unpublished data).

Since the current state of knowledge on the regulation of lipid metabolism by garcinoic acid is based only on cellular models, it is difficult to draw conclusions whether these observed effects may have an influence on in vivo models. As mentioned before, garcinoic acid shows functions similar to other natural compounds such as resveratrol, especially with regard to its antioxidative properties. For this reason, resveratrol is preferred for deducing possible in vivo effects of garcinoic acid on lipid homeostasis. Independent experiments in THP-1 and 3T3-L1 cells showed that CD36 expression is upregulated by resveratrol [192,193]. Unfortunately the uptake of oxLDL has not been measured in these cell models. In addition to the mentioned in vitro studies, Chen and coworkers investigated the effect of resveratrol treatment on lipid homeostasis in skeletal muscles of rats fed a high-fat diet. After 8 weeks of high-fat feeding, the basal CD36 mRNA expression was increased in the intervention group in comparison to controls. The treatment with resveratrol led to a further induction of CD36 expression [194]. Based on this observation it was quite surprising that the enhanced expression of an important lipid importer did not lead to increased intracellular lipid accumulation, indicating that the induction of CD36 expression by resveratrol has no negative effect on in vivo lipid balance [194]. Because of the similarities between the properties of resveratrol and garcinoic acid, it could be hypothesized that a possible upregulation of CD36 expression by garcinoic acid will also have no negative

effects on lipid metabolism in vivo. However, further experiments are needed to prove this concept.

CONCLUSIONS AND PERSPECTIVES

With the evidence of circulating α-LCM in human blood, a new perspective in vitamin E research was presented. In addition to the well-studied TOH and the latterly more focused T3, their LCM must be taken into account to correctly interpret the effects of vitamin E in humans. We speculate that the LCM comprise a new class of regulatory molecules that complicate the interpretation of studies on the effects of vitamin E in vivo as these molecules exert effects that are different from their metabolic precursors. So far, only a few studies have focused on this class of compounds. However, the LCM seem to share properties with their precursors but to exert also unique or even adverse effects. It is evident that the LCM and precursors act in the same manner with respect to cytotoxicity and modulation of COX2 and 5-LOX activity but it is of note that the LCM are significantly more potent than their precursors in these cases. Hence, the LCM may indeed play a role in mediating these effects of vitamin E in the human body although the blood concentrations are significantly lower than those of TOH. In addition, the LCM exhibit different effects, like their prooxidative capacity reported by Birringer et al. [87]. This in turn is surprising, as vitamin E in general is well known for its antioxidative properties. Moreover, the LCM apparently upregulate CD36, while the downregulation of this receptor by TOH has been shown repeatedly. Furthermore, the LCM can act in areas where the TOH are virtually not effective. A prime example is the regulation of COX2 expression (for more information, the reader is referred to the section "Bioactivity of Garcinoic Acid, Vitamin E and Long-Chain Metabolites").

The natural product garcinoic acid is structurally related to the α-LCM. However, little is known about its bioactivity (Fig. 9.9). Merely, its antioxidative and antiproliferative potential as well as its inhibition of DNA polymerase β have been examined. Due to the structural similarities to TOH, T3, and the LCM, many, yet unknown, effects of garcinoic acid can be expected, making garcinoic acid an interesting natural product for pharmacologic research itself. Although little is known on the effects of garcinoic acid, *G. kola* nuts have been reported as inter alia antidotal, antiinflammatory, antidiabetic, and hepatoprotective. It is likely that garcinoic acid contributes to these properties as it has strong antiinflammatory and antioxidative properties. First results support this hypothesis, as garcinoic acid has shown antiinflammatory actions via downregulation of COX2 expression. For this reason it will be interesting to see what effects garcinoic acid shows in different cell and animal models. If the proposed beneficial properties shown in Fig. 9.10 come true, garcinoic acid has to be tested in clinically relevant studies in animals and later on humans. This may lead to the transfer of knowledge from folk medicine to modern medicine to cure disease.

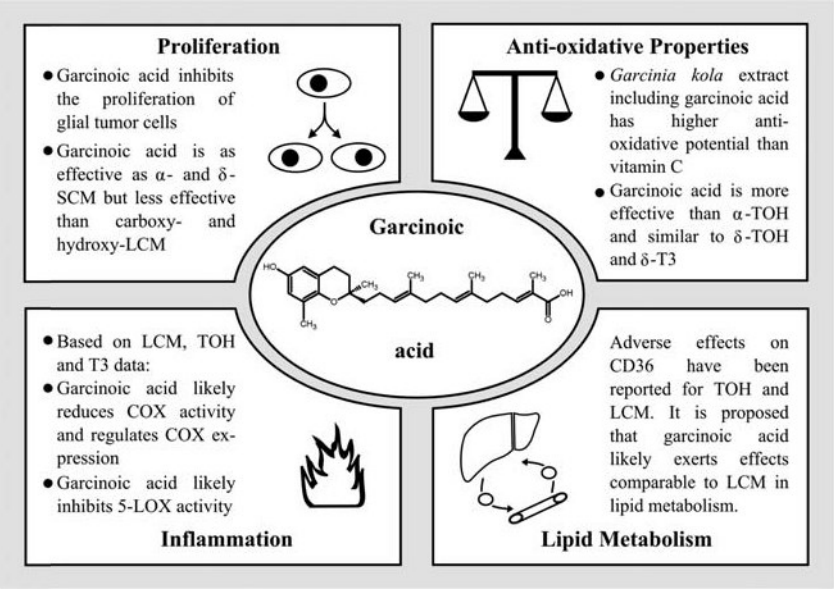

FIGURE 9.9 Known and proposed effects of garcinoic acid.

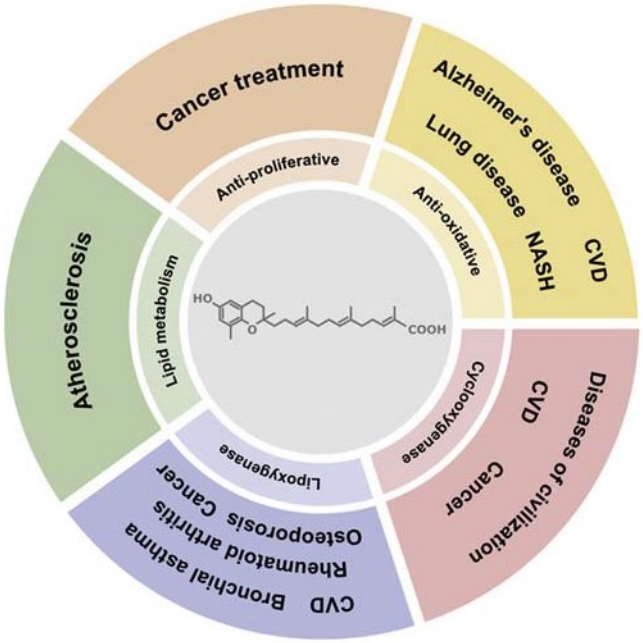

FIGURE 9.10 Proposed beneficial effects of garcinoic acid on human diseases. For detailed information, the reader is referred to the relating chapters. *CVD*, cardiovascular disease; *NASH*, nonalcoholic steatohepatitis.

In addition, garcinoic acid is a helpful substrate that can be reliably isolated from *G. kola* nuts in pure form for synthesizing the δ-LCM, namely δ-13′-OH and δ-13′-COOH, as well as the α-LCM, namely α-13′-OH and α-13′-COOH. The isolation of garcinoic acid from *G. kola* nuts is a simple yet effective method with a yield superior to other reported isolation procedures (the reader is referred to Table 9.1). Thus the procedure provides a reliable base for the synthesis of large amounts of LCM. Pure α- and δ-LCM can be simply and efficiently obtained with the semisynthesis route presented by Mazzini and Birringer. Taken together, this procedure is the most effective way to obtain sufficient amounts of the respective LCM of interest for cellular, animal, as well as human experiments. For this reason, the isolation of garcinoic acid allows the synthesis of the LCM in an elegant and efficient way and the investigation of physiological functions of the α- and δ-LCM in vitro as well as their pharmacological modes of action in vivo in appropriate animal disease models.

To unravel unknown effects and better understand known effects of the different vitamin E forms, as well as to elucidate the underlying regulatory mechanisms that likely involve the LCM, some central questions should be addressed. These include inter alia (1) Which proteins are involved in the uptake and intracellular trafficking of garcinoic acid and of the LCM? (2) Which cellular receptors, signaling proteins, or enzymes mediate the effects of garcinoic acid and of the LCM? (3) What are the regulatory mechanisms that mediate expression of genes in response to garcinoic acid and to the LCM? (4) Which molecular structures are responsible for the effects of garcinoic acid or of the LCM? (5) Do the different LCM differ in their effects and effectiveness? To answer these questions, systematic and comprehensive studies are required. The studies likely involve the identification of potential transporters, binding protein receptors for garcinoic acid, and the LCM. These studies should be complemented by profiling of the effects of garcinoic acid and LCM on gene expression and signaling pathways in different cell types as well as studies in animal models that will shed new light on the regulatory modes of action of the different vitamin E forms and their metabolites. To understand the structure–activity relationship, further structurally related compounds, such as synthetic derivatives of garcinoic acid or of the LCM or enantiomer-pure molecules as well as compounds that represent substructures of the molecule, i.e., the chroman ring or the side chain, should be studied. To sum up, the availability of the LCM as pure compounds provides new perspectives for vitamin E research that will likely contribute to a better understanding of the physiological function of vitamin E. In this respect, the natural product garcinoic acid is a very helpful tool that provides simple and efficient access to the pure α- and δ-LCM for functional studies.

ABBREVIATIONS

13′-COOH 13′-carboxychromanol
13′-OH 13′-hydroxychromanol

5-HPETE	5-hydroperoxyeicosatetraenoic acid
ABCA1	ATP binding cassette transporter A1
ACE	acetone
ACN	acetonitrile
AcOH	acetate
AP-1	activator protein 1
AVED	ataxia with vitamin E deficiency
CC	column chromatography
CD36	cluster of differentiation 36
CEHC	carboxyethyl-hydroxychromanol
cHEX	cyclohexane
CoA	coenzyme A
COX	cyclooxygenase
CPT	centrifugal partition chromatography
CVD	cardiovascular diseases
CYP	cytochrome P450
DCM	dichloromethane
EMSA	electrophoretic mobility shift assays
EtAc	ethyl acetate
HEP	heptane
HEX	hexane
HPLC	high-performance liquid chromatography
ICM	intermediate-chain metabolite(s)
IL	interleukin
iNOS	inducible nitric oxide synthase
LC	liquid chromatography
LCM	long-chain metabolite(s)
LDL	low-density lipoproteins
LOX	lipoxygenase
LPS	lipopolysaccharides
LT	leukotriene
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
MS	mass spectroscopy
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NFκB	nuclear factor "kappa-light-chain-enhancer" of activated B cells
NMR	nuclear magnetic resonance
NPC1L1	Niemann-Pick C1-like protein 1
oxLDL	oxidized LDL
PBMC	peripheral blood mononuclear cells
PGE2	prostaglandin E_2
PKB	protein kinase B (Akt)
PKC	protein kinase C
PUFA	polyunsaturated fatty acid(s)
ROS	reactive oxygen species
SCM	short-chain metabolite(s)
SRB1	scavenger receptor class B type 1

T3	tocotrienol(s)
TCM	chloroform
TLC	thin-layer chromatography
TNFα	tumor necrosis factor α
TOH	tocopherol(s)
TTP	tocopherol transfer protein
VLDL	very-low-density lipoproteins

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7.7 Manuscript VII

antioxidants

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Review

Long-Chain Metabolites of Vitamin E: Metabolic Activation as a General Concept for Lipid-Soluble Vitamins?

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Abstract: Vitamins E, A, D and K comprise the class of lipid-soluble vitamins. For vitamins A and D, a metabolic conversion of precursors to active metabolites has already been described. During the metabolism of vitamin E, the long-chain metabolites (LCMs) 13′-hydroxychromanol (13′-OH) and 13′-carboxychromanol (13′-COOH) are formed by oxidative modification of the side-chain. The occurrence of these metabolites in human serum indicates a physiological relevance. Indeed, effects of the LCMs on lipid metabolism, apoptosis, proliferation and inflammatory actions as well as tocopherol and xenobiotic metabolism have been shown. Interestingly, there are several parallels between the actions of the LCMs of vitamin E and the active metabolites of vitamin A and D. The recent findings that the LCMs exert effects different from that of their precursors support their putative role as regulatory metabolites. Hence, it could be proposed that the mode of action of the LCMs might be mediated by a mechanism similar to vitamin A and D metabolites. If the physiological relevance and this concept of action of the LCMs can be confirmed, a general concept of activation of lipid-soluble vitamins via their metabolites might be deduced.

Keywords: vitamin E; long-chain metabolites of vitamin E; 13′-hydroxychromanol (13′-OH); 13′-carboxychromanol (13′-COOH); vitamin E metabolism; biological activity

1. The Biological Significance of Vitamin E

The term vitamin E comprises eight lipophilic molecules, which can be classified as tocopherols (TOHs) and tocotrienols (T3). Both classes share two common features: (i) the phytyl-like side chain, which is bound to (ii) the chroman ring system. A saturated side chain characterizes the TOHs, while the T3s carry three double bonds in this substructure. Further, the methylation pattern of the chroman ring determines the classification as α-, β-, γ- or δ-TOH or T3, respectively. Vitamin E is found in oils, nuts, germs, seeds and a variety of other plant products. The naturally found vitamin E forms exist either in *RRR*-configuration (TOHs) or in *R*-configuration (T3s), whereas only synthetically produced forms contain a mixture of the different possible stereoisomers [1].

Vitamin E was discovered in 1922 as vital factor for the fertility of rats, indicating its essentiality for animal and human health, and was therefore classified as a vitamin [2]. Nevertheless, the benefits of vitamin E for human health are still a contentious issue. However, several disease conditions, such as anemia, erythrocyte rupture and neuronal degeneration, as well as muscle degeneration, are linked to vitamin E deficiency or malabsorption (extensively reviewed in [3]). Further, vitamin E was shown in human intervention trials to slow down the progression of age-related neurodegenerative pathologies such as Alzheimer's disease, maybe due to its antioxidative properties [4,5]. Vitamin E is also an essential factor for the development of the central nervous system and cognitive functions of the embryo [6,7]. Next, vitamin E may play a supportive role in the prevention of neural tube defects in humans along with folic acid [8,9]. Initially, the effects of vitamin E were only attributed to its antioxidant properties, however more recent work unveiled non-antioxidant regulatory effects. There is growing evidence that vitamin E modulates gene expression and enzyme activities and interferes with signaling cascades independent of its capacity as an antioxidant [10]. Over time, several functions of vitamin E, such as suppression of inflammatory mediators, reactive oxygen species, and adhesion molecules, the induction of scavenger receptors, and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF_kB) (reviewed in [11]) were revealed. Based on these observations, it was concluded that vitamin E likely plays a role in several inflammatory but also other diseases. However, further research is required, as the results obtained from clinical trials with TOHs are inconsistent with respect to beneficial effects on the development of chronic diseases such as cancer and cardiovascular diseases [12].

2. Absorption and Distribution of Vitamin E

Like for all macro- and micronutrients, intestinal absorption is the limiting factor for the bioavailability of vitamin E in humans. As a fat-soluble vitamin, intestinal absorption, hepatic metabolism and cellular uptake of vitamin E follows that of other lipophilic molecules [13]. The absorption rate of vitamin E varies between 20% and 80% [13,14], and is thus generally lower than for vitamins A and D [15,16]. Differences in the rates of absorption of vitamin E and the other fat-soluble vitamins may result also from the parallel intake of additional food ingredients. For example, retinoic acid [17], plant sterols [18], eicosapentaenoic acid [14], alcohol (chronic consumption) [14], and dietary fiber [19] are natural food components that may compete with the absorption of vitamin E. In addition, it has been shown that the supplied form of vitamin E, either as a free molecule or coupled to other compounds like acetate, is also crucial for its bioavailability [20].

For optimal absorption, fat must be consumed along with the ingested vitamin E. This is a general requirement for all types of fat-soluble vitamins and is therefore also applicable for vitamins A, D and K [16,21]. The absorption of triacylglycerides and esterified fat-soluble molecules starts with enzymatic processing in the stomach by the action of gastric lipases [15]. The following digestion of dietary lipids appears in the intestinal lumen by the action of various enzymes, including pancreatic lipase, carboxyl esterase and phospholipase A_2 [22]. Since most of the vitamin E in the human diet is not esterified, lipolytic degradation is scarce [14]. In contrast, the human diet contains significantly more esterified vitamin A and D, mostly in the form of retinyl-esters and vitamin D_3 oleate, which can be hydrolyzed by the above mentioned enzymes [16,21]. A key step of the intestinal absorption of fat-soluble vitamins is the emulsification, i.e., the incorporation into micelles formed with phospholipids and bile acids. Under normal conditions, bile salts facilitate the absorption of all three vitamins, but especially the vitamin D forms differ in their dependency for bile salt availability, i.e., vitamin D_3 absorption is more dependent on the presence of bile salts than 25-hydroxyvitamin D (OHD) [23]. After emulsification, vitamin E is taken up into the intestinal enterocytes by passive diffusion or receptor-mediated transport via scavenger receptor class B type 1 (SRB1) [24], or Niemann–Pick C1-like protein 1 [25], which is also involved in the uptake of the vitamins A, D and K as well as cholesterol [16,26,27]. Since no specific plasma transport protein for α-TOH is known, the subsequent transport of vitamin E in blood follows largely that of cholesterol [25], meaning that under normal

physiological conditions, α-TOH is transported via chylomicrons. This transport is independent of the type of stereoisomer [28,29]. In addition, retinol, unconverted pro-retinoid carotenoids (β-carotene), non-pro-retinoid carotenoids (lycopene), vitamin D_3 and phylloquinone (representing the main dietary form of vitamin K) are also incorporated into chylomicrons [16,21,30]. After entering the circulation, chylomicrons undergo a process of remodeling that involves primarily the hydrolysis of triglycerides by lipoprotein lipase, resulting in the formation of chylomicron remnants [25]. Vitamins E, A, D and K are not affected by hydrolysis and remain in the lipoprotein particle for further transport to the liver [31]. The different forms of vitamin E are discriminated in the liver by the α-tocopherol transfer protein (α-TTP), which promotes the incorporation of 2*R*- or *RRR*-α-TOH into very low-density lipoproteins (VLDL) [32,33], whereas other forms and stereoisomers are either metabolized or secreted into bile [34]. Besides α-TTP, the TOH-associated protein and the TOH-binding protein are known mediators of the intracellular transport of vitamin E. Interestingly, α-TOH secretion from the liver is apparently not necessarily dependent on VLDL assembly and secretion, thus oxysterol-binding proteins [35] and ATP-binding cassette transporter A1 (ABCA1) [36] have been suggested to contribute to the release from the liver. Furthermore, ABCA1 mediates the efflux of vitamin E in the intestine, macrophages, and fibroblasts [36], and multidrug resistance P-glycoprotein has been identified as a transporter for the excretion of α-TOH via bile [37]. After the release of vitamin E-carrying VLDL into blood circulation and action of lipoprotein lipase as well as hepatic lipase, receptors such as SRB1, low-density lipoprotein (LDL) receptor as well as LDL receptor-related protein mediate the uptake of vitamin E into peripheral tissues and the liver [31,38].

3. Metabolism of Vitamin E

The metabolism of vitamin E is primarily localized in the liver (Figure 1) (reviewed in [39]), whereas extrahepatic pathways have been also suggested [40,41]. The degradation processes of hepatic metabolism remain poorly understood, but the initial mechanisms are generally accepted, i.e., all vitamers are degraded to vitamer-specific physiological metabolites with an intact chromanol ring and a shortened side-chain. Interestingly, accumulation of vitamin E to toxic levels is prevented by increased metabolism in response to higher vitamin E levels. Due to the preferential binding to α-TTP, α-TOH is the prevalent form of vitamin E in humans. It is speculated that α-TTP protects the α-form from degradation, thus leading to the accumulation of α-TOH. With the lower affinities of the other vitamin E forms to α-TTP taken into consideration, γ- and δ-forms are likely catabolized faster [42]. Despite of the different catabolic rates, all forms of vitamin E follow the same metabolic route, as confirmed by the detection of the respective end products of hepatic metabolism, α-, β-, γ-, and δ-carboxyethylhydroxychromanol (CEHC) [43,44]. However, the rate of catabolism is different for the vitamin E forms, possibly due to distinct affinities to key enzymes [42,45]. The chroman ring is not modified during catabolism (the catabolic end products are still classified as α-, β-, γ- and δ-forms); it is rather the aliphatic side chain where modifications are introduced. Metabolism of T3 follows the same principle, albeit further enzymes such as 2,4 dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase (necessary for the metabolism of unsaturated fatty acids) are likely required for the degradation of the unsaturated side chain [46].

The catabolism of the vitamin E molecule takes place in different cell compartments: endoplasmic reticulum, peroxisomes, and mitochondria. However, the mechanism of metabolite transfer between the compartments is not well understood and requires further investigation. The initial step at the endoplasmic reticulum leads to the formation of 13′-hydroxychromanol (13′-OH) metabolites via ω-hydroxylation by cytochrome P450 (CYP) 4F2 or CYP3A4, respectively [45,47]. The following ω-oxidation, which is probably mediated by alcohol and aldehyde dehydrogenases (an aldehyde intermediate is formed), results in 13′-carboxychromanol (COOH) metabolites. In general, the resulting metabolites with carboxy function are degraded like branched-chain fatty acids. Hence, the side chain is shortened by β-oxidation, and the formed propionyl-CoA or acetyl-CoA is eliminated. The intermediate-chain metabolites 11′-COOH and 9′-COOH are formed in peroxisomes during

the first two cycles of β-oxidation. Three additional cycles of β-oxidation are carried out in the mitochondria, resulting in the short-chain metabolites (SCMs) 7′-COOH and 5′-COOH as well as the end-product CEHC or 3′-COOH. Moreover, conjugation of the metabolites takes place during metabolism, resulting predominantly in sulfated and glucuronidated metabolites. However, glycine-, glycine–glucuronide-, and taurine-modified metabolites of vitamin E have also been identified [48].

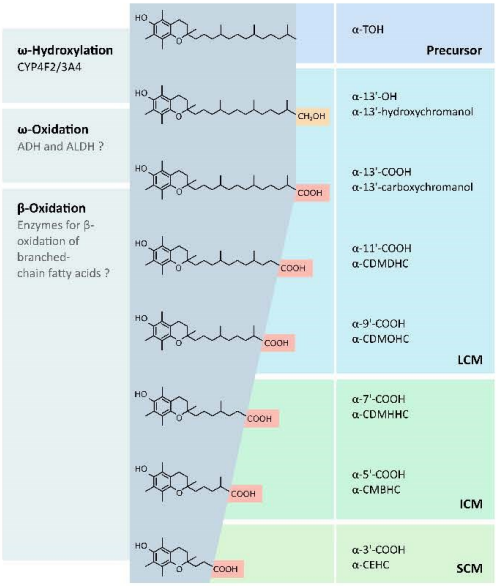

Figure 1. Metabolism of vitamin E. The metabolism of vitamin E is initiated by a terminal ω-hydroxylation of the side-chain via CYP4F2 and CYP3A4. The resulting hydroxychromanol is further modified by ω-oxidation, resulting in the formation of carboxychromanol, possibly by alcohol and aldehyde dehydrogenases. As a consequence, the metabolite can be subjected to β-oxidation. Five cycles of β-oxidation lead to the formation of the short-chain metabolite CEHC. However, this review focuses on the LCMs 13′-OH and 13′-COOH as these molecules have been synthesized in sufficient amounts for in vitro and in vivo investigations. The following abbreviations are used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CDMDHC, carboxydimethyldecylhydroxychromanol; CDMOHC, carboxymethyloctylhydroxychromanol; CDMHHC, carboxymethylhexylhydroxychromanol; CMBHC, carboxymethylbutylhydroxychromanol; CEHC, carboxyethylhydroxychromanol.

The conjugated SCMs are more hydrophilic and thus mainly found in glucuronidated form in human urine [44]. In contrast, the long-chain metabolites (LCMs) and their metabolic precursors are secreted via bile into the intestine and the metabolites in fecal samples are not conjugated. The fecal route is considered as the major pathway of vitamin E excretion [12,49].

Like vitamin E, other fat-soluble vitamins, such as the vitamins A (i), D (ii) and K (iii) are also metabolized in the human body:

(i). Under physiological conditions, retinyl esters (in the intestinal lumen) and carotenoids (in enterocytes) are converted into retinol before or during their intestinal absorption, respectively. Inside the enterocytes, retinol is re-esterified by lecithin-retinol acyl transferase or acyl-CoA:retinol-acyltransferase and packed into chylomicrons for transport. The retinyl esters are transferred to the liver and stored in hepatic parenchymal and non-parenchymal cells.

Vitamin A is mobilized from liver stores by the retinol-binding protein, a specific transporter allowing the transport of retinol in blood circulation [50]. These results suggest that vitamin A has an active (retinol) and a storage form (retinyl ester). In addition, the oxidation of retinol leads to the formation of retinal, another active form of vitamin A, which is primarily bound to opsins in the photoreceptors of the retina [51]. More current research indicates that all-*trans* retinoic acid (ATRA), 9-*cis*-RA, and all-*trans*-4-oxo-RA are the vitamin A metabolites with the highest biological activity. These active vitamin A metabolites serve as ligands for nuclear receptors, called retinoic acid receptors (RARs) [52] and retinoid receptors (RXRs) [53], which act as ligand-activated transcription factors controlling the expression of their respective target genes. Therefore, hepatic retinol is transferred to extrahepatic tissues and metabolized to retinoic acid by different enzymatic systems. LAMPEN and co-workers found that ATRA is also formed in the small intestine via direct oxidation of vitamin A. Based on this result, they hypothesized that biologically active retinoids are formed in the gastrointestinal tract and act as retinoid-receptor ligands controlling various processes in the intestinal mucosa via RAR [53].

(ii). The human metabolism of vitamin D is primarily located in liver and kidney. Metabolism of vitamin D_2 and D_3 starts with the formation of 25-OHD, the major circulating vitamin D metabolite, by vitamin D-25 hydroxylase. Afterwards, 25-OHD is transferred to the kidney and further catabolized by 25-OHD-1α-hydroxylase to 1,25-dihydroxyvitamin $D_{2/3}$. These molecules serve as ligands for the vitamin D receptor (VDR), a transcription factor expressed in various tissues. Vitamin D receptor binds to specific regions in the promoter regions of genes, the so-called vitamin D responsive elements, thus controlling the expression of respective target genes. Therefore, 1,25-dihydroxyvitamin D is the active metabolic form of vitamin D [54,55].

(iii). Phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) are summarized by the term vitamin K. Phylloquinone is synthesized in plants, while menaquinone is derived from animal and bacterial origins [30,56]. Both compounds share a 2-methyl-1,4-naphthoquinone structure, called menadione, and a side chain at the 3′-position. The side chain of phylloquinone is composed of three isopentyl units and one isopentenyl unit, while the side chain of menaquinone contains a variable number of only isopentenyl units (2–13) [30]. The metabolism of vitamin K is localized in the liver and has not been studied in detail so far [57]. Nevertheless, the metabolic pathway of phylloquinone and menaquinone degradation likely follows that of vitamin E. Hence, the degradation starts with an initial ω-oxidation, which is mediated by CYP. While the ω-oxidation of vitamin E is catalyzed primarily by CYP4F2, CYP3A4 has been described as the possible mediator for the ω-oxidation of vitamin K. Next, the following degradation of the side chain of vitamin K occurs via β-oxidation [30,56,58]. A 5-carbon carboxylic acid metabolite termed K acid 2 has been identified as the end-product of either phylloquinone or menaquinone metabolism and is excreted via urine and bile [30,58]. In addition to their metabolic degradation, it has been suggested that phylloquinones could also be converted to menaquinones [59,60]. For this, phylloquinone is likely transformed to the intermediate menadione by removing its side chain, which is subsequently replaced by a newly synthesized isopentenyl side chain to form menaquinone [30]. While menaquinone is considered as the physiologically active form of vitamin K in humans [56], almost nothing is known about a possible biological activity of the vitamin K metabolites. Further studies are needed to unravel whether vitamin K must be included into the general concept of a metabolic pre-activation of lipid-soluble vitamins.

Although the metabolisms of vitamin A and D differ in location and the involved enzymatic systems, the formation of active metabolites seems to be a key element of both metabolic pathways, i.e., both vitamins mediate their gene regulatory effects by metabolic pre-activation. Therefore, the discovery of vitamin E metabolism in animals and humans and the emerging evidence for important biological functions of vitamin E metabolites could indicate a general metabolic activation mechanism of fat-soluble vitamins in the human body.

In Vivo Verification of Systemic LCM Availability

Since the discovery of vitamin E by EVANS and BISHOP in 1922 [2], α-TOH has been accounted as an antioxidant capable to scavenge reactive oxygen species, and decreased α-TOH levels have been associated with several diseases including different types of cancer, cardiovascular diseases and diabetes [61]. It took 80 years since AZZI and co-workers set up the hypothesis for an additional gene regulatory role of α-TOH in the human body [62]. In addition, the discovery of vitamin E metabolism in animals and humans and the emerging evidence for important biological functions of the vitamin E metabolites [63,64], suggested that the TOHs may gain biological activity after metabolism (as confirmed for vitamin A and D). This prompted studies that investigated also the putative functions of the LCMs of TOH. In 2014, Wallert and co-workers showed the occurrence of α-13′-COOH in human serum, which has been confirmed later by others [65,66]. For these studies, serum obtained from a healthy, middle-aged (39 years), non-smoking male, who received a balanced diet with no additional vitamin E supplementation was used for the detection of α-13′-COOH via liquid chromatography coupled mass spectrometry [63]. The analyses revealed for the first time that α-TOH metabolites are transferred into blood circulation following metabolism of α-TOH in the liver. Furthermore, cell experiments showed that α-13′-OH and α-13′-COOH are more potent regulators of gene expression than their metabolic precursor α-TOH [63]. Taken together, the results of Wallert et al. provided the first evidence that the LCMs are an active form of their metabolic precursor [63], promoting regulatory effects in peripheral tissues of the human body. However, while the role of vitamin E as a lipophilic antioxidant in vitro is widely accepted, the relevance in vivo is still a matter of debate [67–69].

4. Biological Activity

Not much is known about the biological activity of the LCMs. However, the publications on this topic published during the last ten years can be categorized by the biological effects of the LCMs as follows: (i) anti-inflammatory actions [64,70–75]; (ii) anti-carcinogenic effects [72,76,77]; (iii) regulation of cellular lipid homeostasis [63,64]; (iv) interaction with pharmaceuticals [78]; and (v) regulation of their own metabolism [79] (Figure 2).

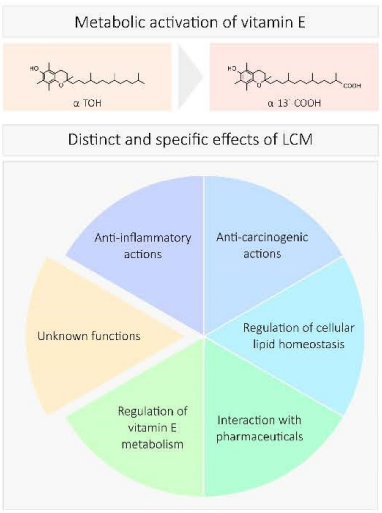

Figure 2. Reported biological functions of the LCMs of vitamin E.

4.1. Anti-Inflammatory Actions

Investigations on anti-inflammatory actions often focus on the regulation of pro-inflammatory enzymes, such as inducible cyclooxygenase 2 (COX2) [70–72,74], inducible nitric oxide synthase (iNOS or nitric oxide synthase, NOS2) [64,71,74,75], or 5-lipoxygenase (5-LO) [72,73], as well as mediators such as chemokines or cytokines. For this purpose, cells were treated with the LCMs and challenged with a pro-inflammatory stimulus or alternatively, isolated enzymes were used. Several LCMs (α-, γ-, δ-13′-COOH; δ-9′-COOH; α-13′-OH) have been tested and reduced the stimulus-induced expression (mRNA or protein) or enzyme activity. In general, 13′-COOH are more potent than the shorter LCMs and the conjugation of LCMs with sulfate abrogates their anti-inflammatory effects [64,70].

Jiang et al. gained first hints on the anti-inflammatory actions of LCMs [70]. A549 cells, which are capable of metabolizing vitamin E, were incubated with TOHs and an inhibition of the arachidonic acid-stimulated COX activity was reported. When the metabolism of vitamin E was suppressed by sesamin, the effects were less pronounced, indicating the involvement of the LCMs as regulatory molecules. For further experiments, the LCMs were extracted from the cell culture medium and their inhibitory capacity on COX activity was tested (half maximal inhibitory concentration (IC_{50}): δ-13′-COOH: 4 μM; δ-9′-COOH: 6 μM). The impact of conjugation was tested, and the sulfate LCM conjugates were unable to exert anti-inflammatory effects. In 2016, a comparison of the different types of LCMs was performed, and the LCMs showed similar effects regardless of their origin (isolated from cell culture medium or semisynthetic isolation from *Garcinia kola*) [72]. In RAW264.7 macrophages, the anti-inflammatory action on lipopolysaccharide (LPS)-stimulated COX2 mRNA and protein expression, as well as prostaglandin (PG) release was reported for α-13′-OH [71] and α-13′-COOH [74].

The regulation of iNos by the LCMs was studied in RAW264.7 macrophages [64,71,74,75]. The LPS-stimulated iNos mRNA and protein expression as well as release of nitric oxide were reduced by the LCMs tested (α- and δ-13′-OH, α- and δ-13′-COOH) [64]. The inhibitory effect of the LCMs was highly dependent on the structure of the LCMs. The 13′-COOH were more effective than the 13′-OH, while the substitution of the chromanol ring system (α- vs. δ-LCMs) had no influence.

The inhibition of ionophore-induced leukotriene release (leukotriene B_4) in HL-60 cells and neutrophils was reported with IC_{50} values of 4–7 μM [73]. Furthermore, the activity of isolated 5-LO was inhibited by δ-13′-COOH with IC_{50} values of 0.5–1 μM, which is more effective than the synthetic 5-LO inhibitor zileuton (IC_{50}: 3–5 μM) [73]. The inhibition of 5-LO activity by δ-13′-COOH was also confirmed by Jang et al. [72]. An overview of the known anti-inflammatory actions of the different LCMs of vitamin E studied so far is provided in Table 1.

Table 1. Overview of anti-inflammatory actions of the LCMs of vitamin E.

Targets	Cells	Effects	Substances	Refs.
COX2	A549 cells	Reduced activity in arachidonic acid-pre-induced cells	γ-13′-COOH δ-13′-COOH δ-9′-COOH	[70] [70,72] [70]
	Isolated enzyme	Inhibition of activity	δ-13′-COOH δ-9′-COOH	[70]
	RAW264.7	Inhibition of LPS-stimulated mRNA and protein expression, as well as reduced PG release	α-13′-OH α-13′-COOH	[71] [74]
iNos	RAW264.7	Inhibition of LPS-stimulated mRNA and protein expression, as well as reduced release of nitric oxide	α-13′-OH α-13′-COOH δ-13′-OH δ-13′-COOH	[64,71,74,75]
5-LO	Isolated enzyme HL-60 neutrophils	Inhibition of activity Reduced activity and LT release in pre-induced cells	δ-13′-COOH δ-13′-COOH	[72,73] [73]

PG, prostaglandin; LT, leukotriene.

The metabolites of vitamin K have also been shown to exert anti-inflammatory functions. First experiments were carried out with a synthetic 7-carbon carboxylic acid vitamin K metabolite (2-methyl, 3-(2′methyl)-hexanoic acid-1,4-naphthoquinone; K acid 1), which was a more effective

inhibitor of LPS-induced IL-6 release from fibroblast than the precursors phylloquinone and menaquinon-4 [80]. In LPS-challenged MG63 osteoblasts the 7-carbon carboxylic acid metabolite as well as the 5-carbon carboxylic acid metabolite (K acid 2) attenuated the expression of IL-6 [81]. Later, the long-chain metabolites of vitamin K (10 to 20-carbon carboxylic acid metabolites) were also synthesized and examined for their anti-inflammatory activity. In LPS-challenged mouse macrophages, these compounds reduced the induction of gene-expression of the inflammatory markers IL-1β, IL-6 and TNFα [82]. However, K acid 1 and K acid 2 were also effective in this study; and it is not possible to estimate, which vitamin K metabolite (either long-chain or short-chain) is the most effective [82]. Interestingly, the minor 7-carbon carboxylic acid metabolite was more effective in MG63 osteoblasts than the 5-carbon carboxylic acid metabolite, and a replacement of the carboxy function by a methyl group made the two metabolites less effective [81]. This is in line with findings for the LCMs of vitamin E. Here, the carboxy metabolite is more effective than the respective TOH precursor with respect to the anti-inflammatory actions (vide supra). However, the in vivo relevance of the regulatory activities of the vitamin K metabolites is a matter of debate, as they increase with vitamin K intake in urine [83], but have not yet been found in human blood or other tissues to the best of our knowledge.

4.2. *Cancerogenesis and Chemoprevention*

The metabolites of vitamin E were investigated with respect to putative anti-cancerogenic, i.e., anti-proliferative and pro-apoptotic, properties in several studies. First experiments revealed that the SCMs inhibit cell proliferation in different cell lines [84,85]. Interestingly, the metabolites as well as the precursor molecules showed different efficiencies, depending on the methylation pattern of the chroman ring and also on the cell type tested [84,85]. Based on the anti-proliferative effects of the SCMs, the interest in the effects of the LCMs aroused. Hence, Birringer et al. investigated the effects of the LCMs α-13′-COOH and δ-13′-COOH as well as α-13′-OH and δ-13′-OH on the proliferation of the human hepatocyte carcinoma cell line HepG2 [77]. Interestingly, both 13′-COOH metabolites effectively caused cell growth arrest, but the hydroxy metabolites did not exhibit anti-proliferative effects. Thus, the introduction of the carboxy group during TOH metabolism renders the molecule active with respect to cell growth arrest. This is supported by the finding that the metabolic precursors, i.e., TOHs, did not affect proliferation of HepG2 cells [77]. As mentioned above, the methylation of the chroman ring alters the efficiency of the molecules. With an effective concentration of 6.5 μM in HepG2 cells regarding the effects on cell growth, the δ-metabolite is more effective than its α-counterpart with 13.5 μM [77]. At first glance, contradictory results were reported for human prostate cancer cells. Here, not only δ-13′-COOH inhibited cell proliferation, but also the hydroxy metabolite α-13′-OH. The LCMs as well as the tested SCMs α-CEHC and γ-CEHC inhibited the proliferation by about 60% in a concentration of 10 μM [76]. Hence, the efficiency of the hydroxy metabolite is likely dependent on the cell type. It is possible that the differences in TOH metabolism in different cell types lead to divergent effects. Interestingly, even differences between different cancer and non-cancer cell lines have been described. The proliferation of the colon cancer cell lines HCT-116 and HT-29 was inhibited by δ-13′-COOH, with IC_{50} values of 8.9 μM and 8.6 μM, respectively [72]. While 10 μM of the LCMs reduce the viability of the cancer cells by around 60%, normal colon epithelial cells showed a reduction of 10–20% at this concentration. Comparable effects were found for the δ-T3 LCM δ-T3-13′-COOH (δ-garcinoic acid), which reduced the viability of the colon cancer cells by about 75%, but the viability of normal colon cells merely by 10–20% [72].

The actions of the vitamin E metabolites are comparable to that of the metabolites of vitamin D and vitamin A. The active vitamin D metabolite $1,25(OH)_2D_3$ has been shown to modulate differentiation and proliferation of colon cancer cells and prostate cancer cells [86]. However, $1,25(OH)_2D_3$ led to an arrest of most cells that express a functional vitamin D receptor in G0/G1 phase [87]. The actions are mediated by interference with several regulatory proteins, such as epidermal growth factor receptor (EGFR), insulin-like growth factors (IGFs), p21, p27 as well as cyclins and cyclin-dependent kinases (CDKs) [87]. The retinoids are also known for their modulation of the cell cycle. In several cancer cell

lines, retinoic acid (RA) led to a cell cycle arrest in the G0/G1 phase via direct or indirect modulation of cyclins, CDKs and cell-cycle inhibitors [88]. Interestingly, TOHs and TOH SCMs have also been linked to cyclins and CDKs. In the human prostate cancer cell line PC3, γ-TOH as well as γ-CEHC led to a strong decrease in cyclin D1 protein expression. In line with this observation, CDK4 and p27 expression are reduced, albeit less pronounced [85]. Moreover, α-TOH and α-CEHC are ineffective with respect to anti-proliferative actions as well as suppression of cyclin D1 and CDK4 [85]. However, to date, no data is available on the action of the vitamin E LCMs on cell cycle regulators, although strong anti-proliferative effects have been shown for this class of metabolites.

More detailed investigations were carried out on the pro-apoptotic effects of the vitamin E LCMs. Birringer et al. found a significant induction of apoptosis in HepG2 cells treated with 20 μM of α-13′-COOH, δ-13′-COOH or δ-13′-OH [77]. The LCMs induced the cleavage of caspases 3, 7 and 9, and in line with this, the cleavage of the downstream mediator poly-ADP ribose polymerase-1 (PARP-1). Again, the 13′-COOH were more effective in caspase-cleavage and apoptosis induction than the hydroxy metabolite [77]. Moreover, induction of mitochondrial apoptosis by the LCMs was identified as the process leading to apoptosis. This process is accompanied by the formation of reactive oxygen species (ROS). Birringer et al. observed a significant increase in ROS production in cells treated with α- and δ-13′-COOH but not with the hydroxy metabolites and the TOHs [77]. The augmented ROS production was not only measured intracellularly but also intramitochondrial, hence providing evidence for mitochondrial-derived apoptosis. Alterations in the mitochondrial membrane potential supported this finding. Treatment with 20 μM of the LCMs led to a significant reduction of the mitochondrial membrane potential. Interestingly, in this particular case, the α-metabolite was more potent than the δ-metabolites with 60% reduction vs. 20% reduction [77]. The pro-apoptotic actions of the δ-LCMs of vitamin E were confirmed in colon cancer cells [72]. Early and late apoptosis were induced by δ-13′-COOH and δ-T3-13′-COOH. The activation of caspase-9 and cleavage of PARP found by Birringer et al. [77] were confirmed in colon cancer cells [72]. Moreover, an induction of the autophagy marker microtubule-associated protein 1A/1B-light chain 3 (LC3)-II was found. Jang et al. assumed that alterations in sphingolipid metabolism caused by the carboxy-LCMs are the reason for the induction of apoptosis. Indeed, both δ-13′-COOH and δ-T3-13′-COOH increased total ceramides, dihydroceramides and dihydrosphingosines, while all measured sphingomyelins were decreased. Inhibition of sphingosine biosynthesis revealed that LC3-II expression but not PARP-cleavage is modulated by the LCMs via alterations in sphingolipid metabolism [72].

Taken together, there are several similarities between the metabolites of vitamins A, D and E with respect to anti-cancerogenic properties. Data on anti-cancerogenic effects of vitamin K metabolites, however, are sparse. Merely synthetic carboxylic derivatives of menaquinone with different side-chain lengths have been studied [89]. The biologically most abundant 5-carbon carboxylic acid metabolite (K acid 2) was not included in this study and the 7-carbon carboxylic acid metabolite (K acid 1) was the structure with the shortest side-chain. Interestingly, the growth-suppressing effect on hepatocellular carcinoma cells increased with the length of the side chain of the carboxy derivatives, except for the full-length metabolite, which was as effective as the 7-carbon carboxylic acid metabolite. Conversely, menaquinone itself was completely ineffective, showing nicely that the introduction of a carboxy function activates the compound. Blocking of the effects with chemical antagonists suggested that the derivatives act through caspase/transglutaminase-related signaling [89]. The above mentioned disruption of mitochondrial function by the LCMs of vitamin E has also been described for the metabolites of vitamin A [90], and induction of apoptosis by $1,25(OH)_2D_3$ via mitochondrial pathways (e.g., via B-cell lymphoma (BCL)-2 and BCL-xL) in breast, colon and prostate cancer cells are also known [87]. Based on their anti-proliferative and pro-differentiation actions but also due to the induction of cell death, retinoids are used for treating certain types of cancer [91]. Vitamin A metabolites were successfully used in the treatment of acute promyelocytic leukemia (ATRA and 13-*cis*-RA, 13*c*RA), squamous cell skin cancer and neuroblastoma (13*c*RA), lung cancer (ATRA) and Kaposi's sarcoma (9-*cis*-RA, 9*c*RA). Beneficial effects of retinoids in cancer prevention have also been observed.

These properties can be explained by the targeting of regulators of cell cycle progression by retinoids. The expression of the CDK inhibitors p21 and p27 is regulated by ATRA via RARβ2 upregulation, and retinoic acid has been shown to stimulate the degradation of cyclin D1, leading to a suppression of CDK activity [91]. Interestingly, TOHs as well as SCMs of vitamin E modulate cyclins, CDKs and CDK inhibitors [85]. Albeit the LCMs of vitamin E efficiently suppress proliferation, the identification of effects on regulators of cell cycle progression is pending. However, given that 'decreased proliferation is one of the best biomarkers of a cancer preventive effect' [91], vitamin E and its metabolites are promising compounds for cancer prevention.

4.3. Cellular Lipid Homeostasis

To date, the effects of the LCMs of vitamin E on cellular lipid homeostasis have not been investigated extensively. However, the regulation of key metabolic pathways in foam cell development of macrophages by the LCMs were of particular interest in a study by Wallert et al. [63]. Here, the regulation of the expression of the cluster of differentiation 36 (CD36), the uptake of oxidized low density lipoprotein (oxLDL), phagocytosis and the intracellular storage of lipids were investigated [63]. For this, the monocytic THP-1 cell line, which can be differentiated to macrophage-like cells, was used. In differentiated macrophages, the LCMs α-13′-OH and α-13′-COOH induced the expression of CD36 mRNA and consequently CD36 protein levels. In contrast, the precursor α-TOH exerted opposite effects on CD36 mRNA and protein. Whereas α-TOH reduced the expression of CD36 at a concentration of 100 μM, the α-LCMs induced the expression of CD36 in concentrations of 5 and 10 μM, respectively [63]. Thus, the α-LCMs not only act in a different way than their precursors, but appeared to be also significantly more potent. Interestingly, similar effects were described for the lipid soluble vitamin A. Langmann et al. found that the precursor β-carotene is less effective in inducing expression of CD36 than its metabolites ATRA and 9*c*RA in human monocytes and macrophages [92]. The authors stated that the metabolites 9*c*RA and ATRA displayed high biological activity [92], while the precursors retinol and β-carotene were only marginally metabolized, an observation that parallels the characteristics of the LCMs of vitamin E with respect to their reported serum concentrations [63,93]. The effects of vitamin A metabolites are better characterized than that of the LCMs of vitamin E. It was repeatedly shown that the metabolites of vitamin A regulate CD36 expression in macrophage cell models. The metabolite 9*c*RA induced CD36 mRNA [94,95] and protein expression [95] in human THP-1 macrophages. ATRA increases expression of CD36 mRNA in THP-1 cells [96] and CD36 protein in THP-1 and HL60 macrophages [96,97]. The induction of CD36 expression by ATRA and 9*c*RA has been confirmed in primary human monocytes and macrophages [92,96] to show the physiological relevance in non-cancer cells. With the same intention, it was also shown that the LCMs of vitamin E induced CD36 expression in peripheral blood mononuclear cell (PBMC)-derived primary human macrophages [63].

The scavenger receptor CD36 mediates the uptake of the modified lipoprotein oxLDL [98], a process that in turn stimulates CD36 expression [99]. Given the induction of the expression of CD36 by the LCMs of vitamin E under basal conditions (vide supra), a further stimulation by oxLDL treatment could be expected. As the uptake of oxLDL is a hallmark of macrophage foam cell formation, Wallert et al. examined whether preincubation of THP-1 macrophages with the LCMs of vitamin E affects the oxLDL-induced expression of CD36 [63]. As expected, CD36 expression was induced by oxLDL treatment. Pre-treatment with α-TOH suppressed the induction by oxLDL. In contrast, the pre-incubation with the LCMs augmented the induction of CD36 expression by oxLDL. These findings resemble the reaction of the cells in the absence of oxLDL to α-TOH and its LCMs. Given the higher CD36 expression in the presence of the LCMs, the uptake of oxLDL should in turn be induced in LCM-treated macrophages. However, pre-incubation of the macrophages with the LCMs for 24 h led to decreased oxLDL uptake. Incubation with both, α-13′-OH or α-13′-COOH, decreased the uptake by about 20%. This effect was again confirmed in PBMC-derived macrophages. Here, oxLDL uptake was decreased by α-13′-OH pre-treatment by 24% and by α-13′-COOH pre-treatment

by 20%, respectively [63]. The LCMs of vitamin E thus exerted unexpected effects on oxLDL uptake. As mentioned before, vitamin A metabolites also caused increased CD36 expression, but the metabolite 9*c*RA induced the binding and uptake of oxLDL in THP-1 macrophages as expected [94]. Generally, an activation of RXR leads to an augmented association of oxLDL to THP-1 macrophages [100]. However, 9*c*RA also promoted the degradation of oxLDL and the cholesterol efflux via ATP binding cassette transporters, thus leading to a net depletion of cholesterol esters. Triglyceride levels were apparently not affected, neither by oxLDL treatment nor combination with 9*c*RA [94]. In contrast, in the study of Wallert et al. on the LCMs of vitamin E, oxLDL treatment of the macrophages led to an increase of neutral lipids in the cells. Preincubation with the LCMs diminished the oxLDL-induced neutral lipid accumulation [63]. However, the contradictory results on the effects of the LCMs on CD36 expression and oxLDL uptake required an alternative explanation how the LCMs decrease oxLDL uptake. Thus, Wallert et al. focused on phagocytosis as an alternative uptake mechanism for oxLDL [101]. Indeed, treatment of the macrophages with α-13′-OH led to an inhibition of phagocytotic activity of 16% and with α-13′-COOH of 41%, respectively [63]. Hence, the inhibition of phagocytosis by the LCMs might explain the discrepancy between their effects on CD36 expression and oxLDL uptake in this study.

Taken together, the metabolites of vitamin E and vitamin A induce the expression of CD36 in macrophages. However, their effects on oxLDL uptake are different. While the vitamin A metabolite 9*c*RA induces oxLDL uptake, the LCMs of vitamin E reduce it. In contrast to vitamin A and vitamin E metabolites, the metabolite of vitamin D, 1,25$(OH)_2D_3$ has been shown to reduce the expression of CD36 mRNA and protein in oxLDL-treated macrophages obtained from diabetic subjects. Concomitantly, oxLDL and cholesterol uptake are decreased [102,103]. Hence, the vitamin D metabolite as well as the vitamin E LCMs suppress macrophage foam cell formation and may thus exert positive effects in the context of atherosclerosis prevention.

4.4. Interaction with Pharmaceuticals

The interaction of the vitamin E LCMs with pharmaceuticals was tested by analyzing the regulation of P-glycoprotein (P-gp). P-gp regulates, inter alia, the intracellular concentration of pharmaceuticals and its expression is regulated by various transcription factors, including heat shock transcription factor 1, nuclear factor Y and the pregnane X receptor (PXR) [104,105].

Several vitamin E forms and their metabolites (α-TOH, α-T3, α-13′-COOH, α-CEHC, γ-TOH, γ-T3, γ-CEHC and plastochromanol-8) were used and the regulation of P-gp expression was analyzed in human epithelial-like colon LS180 cells [78]. Only α-13′-COOH and γ-T3 induced P-gp expression and α-T3, α-13′-COOH as well as γ-T3 induced the activity of PXR in a reporter gene assay. In case of vitamin E supplementation, an interaction with the metabolic handling of pharmaceuticals might be possible.

4.5. Regulation of LCM Formation

The regulatory processes, which modulate the metabolism of vitamin E, are largely unknown. In this context, two key issues are important: (i) Apart from CYP4F2 and CYP3A4, the full set of enzymes involved in the first steps of the catabolism of vitamin E remains to be identified, and (ii) the mechanisms by which vitamin E metabolism is regulated have not yet been sufficiently unraveled. However, the upregulation of CYP4F2 protein expression by α-13′-OH in human HepG2 liver cells was reported recently [79], pointing to a positive regulatory feedback loop. If this concept holds true, the enhancement of metabolism by products would be a new facet for the fat-soluble vitamins, as the metabolism of vitamin A and D is mainly regulated negatively by their metabolic products [54,106].

The aldehyde- and alcohol-dehydrogenases have been suggested to be responsible for the ω-oxidation steps and the enzymes for branched-chain fatty acids might catalyze the subsequent β-oxidation [107]. Following the identification of the specific set of enzymes required for vitamin E

metabolism, a major aim will be the characterization of the regulatory factors, which modulate the metabolism of vitamin E.

5. Structure-Specific Effects

To get deeper insights into the specificity of the regulatory effects of the LCMs of vitamin E, a structure-activity study was conducted [64]. For this purpose, substances were used that represent specific substructures of the LCMs or their precursors. The chromanol ring system was mirrored by the SCM α-CEHC and the modified side-chain was represented by the branched-chain fatty acid pristanic acid. Furthermore, the α- and δ-forms of 13′-OH and 13′-COOH were used to study the influence of the side-chain modification. Overall, the application of α- and δ-forms of LCMs and their precursors (α-TOH, α-13′-OH, α-13′-COOH, δ-TOH, δ-13′-OH, δ-13′-COOH) should clarify the importance of the substitution of the ring-system. The regulation of CD36 and iNos by the test compounds was similar for all of the LCMs, but neither the precursors nor their substructures were able to cause the same effects on the expression of the target genes as the LCMs. The substitution of the chromanol ring system had no influence (α- and δ-forms), while the modification of the side-chain (oxidation of TOH to 13′-OH and 13′-COOH) was highly relevant for the effects. Overall, the 13′-COOH was most potent in this study. Based on these specific regulations the existence of specific regulatory molecular pathways for the LCMs has been suggested.

6. Receptors of Vitamin Metabolites

As indicated above, the lipid-soluble vitamins A and D need a conversion to their active metabolites to exert their effects. These metabolites are either bound intracellularly and transferred to the receptor or directly bind the receptor. The receptors for the vitamin A metabolites, RARs and RXRs, were identified in the late 1980s [108–111]. Evidence for binding proteins for the active vitamin D metabolite 1,25$(OH)_2D_3$ was already provided in the 1970's [112,113]; however, cloning of the human vitamin D receptor also succeeded in the late 1980's [114]. In contrast, no specific receptor for vitamin E and/or its metabolites has been identified yet. Interestingly, the metabolites of vitamin A and D act through nuclear receptors. This class of transcription factors can roughly be divided into more specific and rather unspecific members. The vitamin D receptor can be categorized as a more specific receptor, as it is activated by its endogenous ligand 1,25$(OH)_2D_3$ already at sub-nanomolar concentrations [115,116]. This feature is also shared by steroid hormone receptors (estrogen receptor, androgen receptor, ergosterone receptor, cortisol receptor), the thyroid hormone receptor and RARs. The RARs specifically bind ATRA, and also 9*c*RA with lower affinity [117]. The specificity of the nuclear receptors is mainly determined by the structure of the ligand binding pocket. Specific receptors have a relatively small ligand binding pocket, which allows only a limited number of molecules to interact. In contrast, the so-called adopted orphan receptors have a larger ligand binding pocket, allowing the activation of the receptor by a larger number of ligands [115]. Members of this group are the liver X receptors (LXRs), farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs) and RXRs. The latter have been shown to bind the vitamin A metabolite 9*c*RA [118]. However, it is not entirely accepted that 9*c*RA represents the endogenous ligand for RXR [119]. Nonetheless, the example of 9*c*RA opens the possibility that vitamin metabolites act through highly specific receptors but also through rather unspecific ones.

Following the concept that the LCMs of vitamin E represent biologically active metabolites similar to 1,25$(OH)_2D_3$, ATRA and 9*c*RA, these molecules might also exert their effects through nuclear receptors. Indeed, Podszun et al. reported an activation of PXR by α-13′-COOH in the human colon adenocarcinoma cell line LS180 [78] (for detailed information, the reader is referred to the section 'Interaction with pharmaceuticals'). Interestingly, α-T3 and γ-T3 were also able to activate PXR, while α-TOH and γ-TOH as well as the SCMs α-CEHC and γ-CEHC failed to activate PXR [78]. These findings confirm earlier findings in HepG2 cells only in part. In HepG2 cells transfected with PXR and a CAT (chloramphenicol acetyltransferase) reporter gene, α-T3 and γ-T3 efficiently

activated PXR-mediated gene transcription, but α-TOH, γ-TOH and δ-TOH were also able to induce the expression of the reporter gene via PXR [120]. In contrast, the SCMs α-CEHC and α-CMBHC were not able to activate PXR in this study and the LCMs were not tested [120]. Taken together, the T3s reliably activate PXR but the effects of the TOHs need further investigation. Possibly, LS180 and HepG2 metabolize TOH with different efficiency, in turn determining the amounts of LCMs formed as PXR-activating metabolites. Hence, the observed effects of TOHs in HepG2 might be explained by the intracellular formation of the LCMs. However, further investigations on the cell-type specific metabolism of TOH are needed to confirm this hypothesis. Further, with PXR a rather unspecific nuclear receptor is identified for TOHs and their LCMs. As a general sensor for toxic compounds and xenobiotics, PXR has a large ligand binding cavity, which allows the binding of a wide range of ligands [121]. Thus, it is not surprising that PXR has been described as a receptor of vitamin K [122,123], and it has been reported that several menaquinone derivatives activate PXR [124]. Unfortunately, the biologically occurring carboxy derivatives were not included in this study. Hence, merely speculations about the activity based on structure-function-relationships are possible. A reporter gene assay revealed that a terminal phenyl group enhances the activity of the derivatives, while a terminal hydroxy group diminished it compared to the unmodified menaquinone [124]. In conclusion, a more hydrophobic side chain leads to an increased activity on PXR. Hence, the natural metabolic products in humans bearing a terminal carboxy group are likely less potent with respect to the activation of PXR. However, this concept is in contrast to the findings for vitamin E. The TOH precursors are unable to activate PXR, while the LCM α-13′-COOH activates it [78]. Hence, further studies are needed to clarify whether vitamin K metabolites are physiological ligands for the rather unspecific nuclear receptor PXR, like their metabolic precursor menaquinone and the LCMs of vitamin E.

Given that RXR as a receptor for the vitamin A metabolite 9*c*RA is also rather unspecific, it might be possible that the LCMs of vitamin E also act through PXR. However, it is questionable whether all of the reported biological effects of the LCMs, i.e., anti-inflammatory actions, anti-cancerogenic features, and effects on cellular lipid homeostasis (please refer to the respective sections here) can be ascribed to PXR activation. Hence, further investigations aiming at the identification and characterization of receptors for the LCMs of vitamin E LCMs are highly required. Strategies for the identification of further receptors or a receptor specific for the LCMs of vitamin E might be the use of target fishing approaches, gene expression arrays, knockdown/knockout studies, as well as reporter gene assays and ligand binding studies.

7. Conclusions

With the detection of the LCMs of vitamin E in human serum, an important hint for the possible action of these metabolites as signaling molecules was provided. Several studies reinforced this hypothesis by the characterization of the biological effects of the LCMs, as summarized in Figure 2. Interestingly, the LCMs act more potent and in part even contrary to their metabolic precursors. Some of the controversial effects reported for vitamin E might be therefore explained by the action of the LCMs. The evidence of circulating α-LCM in human blood (nanomolar concentrations) provides a new perspective in vitamin E research [63]. Therefore, the LCMs must be seriously considered to correctly interpret the effects of vitamin E in humans, beside the better studied TOHs and T3s. So far, only a few studies have focused on this class of compounds. However, based on our current knowledge and our studies in progress, we speculate that the LCMs comprise a new class of regulatory molecules. These molecules can exert effects that are different from their metabolic precursors, complicating the interpretation of studies on the effects of vitamin E in vivo. Nevertheless, the LCMs share properties with their precursors but also exert unique or even adverse effects. It is evident that the LCMs and their precursors act in the same manner with respect to the modulation of COX2 and 5-LOX activity, but it is of note that the LCMs are significantly more potent than their precursors. Furthermore, the LCM can act in areas where the TOHs are virtually not effective. A prime example is the regulation of COX2 expression. Hence, the LCMs may indeed play a role in mediating some of the effects of vitamin E

in the human body although blood concentrations are significantly lower than those of TOH. So far, blood concentrations are the only valid value for the systemic distribution of the LCMs of vitamin E in the human body. However, based on preliminary data of unpublished in vitro and in vivo studies of our group, we can hypothesize that the LCMs of vitamin E may also accumulate in different parts of the human body, where they reach concentrations higher than in blood. Further studies are required to study this issue in more detail and to differentiate between physiologic (at low concentrations) and pharmacologic (at high concentrations) actions of the LCMs.

To sum up, the LCMs could be regarded as the metabolically activated forms of vitamin E. This is in line with the metabolic activation of the other lipid-soluble vitamins A and D. Consequently, the concept of metabolic activation established for vitamin A and D could now be extended to vitamin E. Thus, a general concept for the biological activity and modes of action of the lipid-soluble vitamins could be defined.

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7.8 Manuscript VIII

Chapter 1

The Hepatic Fate of Vitamin E

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Additional information is available at the end of the chapter

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Abstract

Vitamin E is a lipophilic vitamin and thus is naturally occurring mainly in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols, tocotrienols, and "vitamin E-related structures." Vitamin E follows the same route in the body like other lipophilic substances. In brief, vitamin E is absorbed in the intestine, packaged into chylomicrons together with other lipophilic molecules, and distributed via lymph and blood in the body. As the liver is the central organ in lipoprotein metabolism, it is also essential for the uptake, distribution, metabolism, and storage of vitamin E. Based on the current knowledge on that field, the physiological, nonphysiological, and pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis, are described.

Keywords: vitamin E, liver, hepatic handling, vitamin E homeostasis, AVED

1. Introduction

Vitamin E is a lipophilic vitamin and thus naturally mainly occurring in high-fat plant products such as oils, nuts, germs, seeds, and in lower amounts in vegetables and some fruits. The term "vitamin E" comprises different structures that are classified as tocopherols (TOH), tocotrienols (T3), and "vitamin E-related structures". However, α-TOH is considered as the most important representative of vitamin E in humans as the central vitamin E metabolizing organ, the liver, discriminates for this form [1]. Notwithstanding the classification as vitamin, the way vitamin E exactly contributes to human health is controversially discussed. Vitamin

E deficiency has been linked to several disease states like ataxia with vitamin E deficiency (AVED) [2, 3] and Alzheimer's disease [4, 5], indicating a role in the preservation of human health. AVED has severe neurological consequences and is caused by a defect in the α-TOH transfer protein (α-TTP); the protein responsible for the discrimination of α-TOH from the other vitamin E forms in the liver [2, 3]. This emphasizes the role of the liver as a central organ in human vitamin E handling. The liver further distributes vitamin E in the body [6] and metabolizes excess vitamin E in order to form products for excretion [6] or presumably to produce activated metabolites of vitamin E as known for other lipophilic vitamins [7]. Given the crucial role of the liver for vitamin E handling, this review aims to summarize the knowledge on the physiological hepatic handling of vitamin E as well as on factors influencing hepatic handling of vitamin E.

2. Physiological hepatic handling of vitamin E

The liver is the central organ of vitamin E handling. While intestinal absorption efficiency is similar for all forms of vitamin E [8], the plasma concentrations of vitamin E forms differ a lot (e.g., 22.1 μM for α-TOH vs. 2.2 μM for γ-TOH [9]). The preference of α-TOH in the human body is mediated by several complex and interacting hepatic mechanisms.

2.1. Hepatocellular uptake of vitamin E

Vitamin E is absorbed in the intestine along with lipids (for details, see [8]) and is packed into lipoproteins. These are transported via lymph or blood toward the liver (via chylomicron remnants, low density lipoproteins (LDL), and high density lipoproteins (HDL) [10, 11]). Different mechanisms facilitate the cellular uptake of vitamin E: (i) via lipid transfer proteins or lipases, (ii) receptor-mediated lipoprotein endocytosis, and (iii) selective lipid uptake [12]. The degradation of chylomicrons to chylomicron remnants by lipoprotein lipase (LPL) seems to be highly important for vitamin E uptake in the liver; when lipolysis of triglyceride-rich chylomicrons by LPL is inhibited, the α-TOH uptake in the liver is diminished [13]. The phospholipid transfer protein (PLTP) mediates the exchange of phospholipids between lipoproteins [14] and is also able to bind α-TOH *in vitro* [15]. PLTP-null mice have lower hepatic levels of vitamin E than the wild-type mice [16]; hence, the transfer of vitamin E between the lipoproteins seems to be important for its effective hepatic uptake. The chylomicron remnants and LDL are taken up by the liver via endocytosis, mainly mediated through the LDL receptor (LDLR) or LDLR-related proteins [6, 17]. In addition, the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) is involved in hepatic vitamin E uptake; α-TOH binds to the N-terminal domain of NPC1L1, which mediates α-TOH uptake via endocytosis (mechanism similar to intestinal cholesterol uptake) [18]. The scavenger receptor B type I (SR-BI) is known to mediate the uptake of vitamin E in several tissues (e.g., intestine [19], epithelium [20], and hepatocytes [21]) by channeling the molecules into the cells (shown for cholesterol or triglycerides [22]). Furthermore, the scavenger receptor cluster of differentiation 36 (CD36) is likely involved in hepatic uptake of vitamin E [23].

2.2. Intracellular trafficking of vitamin E

Following its lipophilic nature, vitamin E is transported by intracellular carrier proteins [24]. The intestinally absorbed vitamin E is taken up via endocytosis [25] and follows endosomal fate. Here, the hepatic sorting of vitamin E forms starts as a specific protein, called α-TTP selectively recognizes and preferentially binds α-TOH, which is then extracted from endosomes and transported to the inner leaflet of the plasma membrane [26]. α-TTP is therefore considered to be a "gatekeeper", which discriminates non-α-TOH forms [27] and regulates the plasma concentrations of α-TOH [1]. The affinity of α-TTP to the different forms of vitamin E differs greatly: it is defined as 100% for α-TOH, whereas β-TOH has 38%, γ-TOH 9%, δ-TOH 2%, and α-tocotrienol (T3) 12% affinity to α-TTP [28]. The regular function of α-TTP is crucial, since missense mutations lead to the disruption of α-TOH distribution and the development of a severe degenerative disease, termed AVED [29]. The transfer of α-TOH from endosomes to the plasma membrane is a multi-step process. First, it is speculated whether the ATP-binding cassette transporter A1 (ABCA1) enriches the outer layer of endosomes with α-TOH [30]. The cholesterol transporter NPC1 may also be involved, as a genetic missense mutation of the *NPC1* gene leads to an accumulation of α-TOH in late endosomes [31]. Second, α-TTP extracts the α-TOH from endosomes, and third, α-TTP mediates its transport to the plasma membrane [24]. This process seems to depend on phosphatidylinositol phosphates (PIPs; preferentially $PI(4,5)P_2$ and $PI(3,4)P_2$) in the plasma membrane, as α-TTP binds to them, in turn targeting α-TOH to the plasma membrane and stimulating its release [32]. Chung et al. analyzed the localization of α-TTP depending on the cellular α-TOH concentration [33]. They found (i) perinuclear localization for α-TOH-depleted cells, (ii) a directional transport of α-TOH/α-TTP toward the plasma membrane, when depleted cells were pulsed with a low dose of α-TOH, and (iii) a homogenous cytosolic pattern under long-term and high-dose treatment of cells with α-TOH, which was suggested to be the picture of several α-TOH transport cycles [33]. Furthermore, the authors also postulated a bi-phasic concentration-dependent circulation of α-TTP: the $PI(4,5)P_2$ gradient (low in endosomes and high in plasma membrane) forces the α-TTP-mediated transport of α-TOH toward the plasma membrane, whereas the α-TOH gradient (low in plasma membrane and high in endosomes) triggers the recycling of α-TTP toward the endosomes [33]. It has been proposed that once α-TOH is incorporated into the plasma membrane, it is mediated toward the outer leaflet of the membrane by a flippase, maybe ABCA1, and is then available for the uptake via very low density lipoproteins (VLDL) [34]. For more details on the process, please see Section 2.5 "Release of vitamin E".

2.3. Intracellular storage of vitamin E

Intracellular storage of vitamin E is limited to the lipophilic sites of the cell, which are membranes and lipid droplets [33]. Not much is known about a specific localization of vitamin E accumulation in liver cells, apart from the observation that lysosomal membranes of rat livers seemed to have the highest concentration of all membranes [35–37]. However, it is known that one-third of the total body vitamin E is stored in the liver [38]. Within membranes, vitamin E is thought to stabilize the membrane bilayers due to colocalization with phosphatidylcholine [39] and cholesterol (leading to an association to lipid rafts) [40]. It was further hypothesized

that vitamin E also colocalizes with poly-unsaturated fatty acids (PUFAs) in nonraft domains in order to provide protection from lipid peroxidation [41]. Newly added α-TOH in cell culture enriches in the same organelles as the endogenous α-TOH pool [42]. Hereby, the subcellular content of α-TOH was directly proportional to the lipid content [43].

Our knowledge about the storage of vitamin E in lipid droplets is also limited. It was recently reported that newly endocytosed vitamin E was also found in lipid droplets, thus indicating endosome-lipid-droplet interactions [33].

2.4. Hepatic metabolism of vitamin E

The hepatic metabolism of vitamin E has not been fully characterized. However, the principle steps of vitamin E degradation, that is, the shortening of the side chain without the alteration of the chroman ring, are generally accepted. Hence, the metabolites are classified as α-, β-, γ-, and δ-metabolites according to their respective precursors.

In principle, TOHs and T3s are degraded like long branched chain fatty acids (TOH) or long unsaturated branched chain fatty acids (T3) via β-oxidation in peroxisomes. However, as TOHs and T3s do not bear a terminal carboxy function in their side chain, they are not susceptible to β-oxidation. Hence, the initial and rate-limiting step in vitamin E degradation is the introduction of a carboxy function to the ω-terminus of the side chain. This first step is carried out in the endoplasmic reticulum (ER) of liver cells [44]. Here, two representatives of the cytochrome P450 (CYP) protein family, namely, CYP4F2 [45] and CYP3A4 [46, 47], have been identified to catalyze the initial ω-hydroxylation step. The resulting 13′-hydroxychromanol (13′-OH) is then further metabolized via ω-oxidation, a step that most likely involves alcohol dehydrogenase and aldehyde dehydrogenase [44], leading to 13′-carboxychromanol (13′-COOH). The carboxylated side chain resembles a long branched chain fatty acid that is further degradable via β-oxidation. However, a transport mechanism for the carboxychromanol from the ER to the peroxisomes has not been identified so far. Nevertheless, two cycles of peroxisomal β-oxidation after the activation of α-13′-COOH to the respective CoA ester have been suggested [44], as the peroxisomal β-oxidation system has a higher affinity toward long branched chain fatty acids than the mitochondrial counterpart [48]. The proposed 11′- and 9′-COOH metabolites have indeed been identified in human and mouse samples [49] as well as in a hepatic cell line [45, 50]. Subsequently, three more cycles of β-oxidation are needed to form the final product of vitamin E degradation, namely, carboxyethyl hydroxychromanol (CEHC) or 3′-COOH. These steps, however, are assigned to mitochondrial β-oxidation, as CEHC has solely been found in the mitochondria of hepatic cells [44]. Again, the transport mechanisms of the long-chain metabolites (LCM) (13′- to 9′-COOHs) from peroxisomes to the mitochondria are not known. The respective products for each cycle of β-oxidation (7′-COOH, 5′-COOH, and 3′-COOH) have been identified in different human and murine tissues [49, 51–54] as well as the hepatic cell line HepG2 [45, 47, 51]. Taken together, the hepatic metabolism of vitamin E is characterized by a series of β-oxidation steps after an initial introduction of a carboxy moiety at the ω-terminus of the phytyl-like side chain. The metabolism likely takes place in different cell compartments depending on the enzymatic systems needed for the different degradation steps. However, a concept of vitamin E degradation exclusively in mitochondria cannot be excluded [44]. T3 degradation is believed to follow the same route as TOH degradation but requiring further steps due to the unsaturated side

chain. In line with this assumption is the identification of the respective unsaturated metabolites from 13′-carboxytrienol down to carboxymethylbutadienylhydroxychromanol (CMBenHC) in human and mouse samples [49]. According to these findings, the side chain of the T3 metabolites needs a saturation step before the shortening of the chain. Enzymes involved in the degradation of unsaturated fatty acids like 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase were suggested to contribute to the degradation of T3s [55].

2.5. Release of vitamin E

Following the nature of the lipoprotein metabolism, hepatic release of vitamin E is mostly realized via VLDL. Thus, this section will focus on the packaging of vitamin E into VLDL particles, notwithstanding that the mechanism is not well understood. However, hepatic transfer of vitamin E to HDL has also been suggested [56]. Since it was shown that the expression of α-TTP is crucial for the maintenance of plasma α-TOH levels [57, 58] and that the liver is controlling plasma α-TOH levels [59], hepatic α-TTP is likely involved in the incorporation of vitamin E into lipoproteins. This concept is supported by the observation that nascent VLDL particles are preferentially enriched with *RRR*-α-TOH after oral administration of vitamin E ([60, 61]. In contrast, in the liver, no preferential retention of *RRR*-α-TOH was found, indicating that α-TTP is not involved in the delivery of vitamin E to the liver, but in the release from the liver [62]. Hence, efforts have been made to identify the intracellular location of VLDL enrichment with α-TOH mediated by α-TTP [30]. According to the assembly of VLDL, either the rough ER or the Golgi apparatus were assumed. However, the action of α-TTP in these compartments was not confirmed as the nascent VLDL particles contained equal amounts of SRR and RRR α-TOH forms [30]. Further, the inhibition of ER/Golgi action in cells overexpressing α-TTP did not prevent α-TOH secretion [63]. In conclusion, α-TTP is necessary for the hepatic release of vitamin E, but the enrichment of VLDL with *RRR*-α-TOH occurs after exocytosis.

Based on this, the hypothesis of α-TOH uptake by VLDL directly from the plasma membrane was developed. This idea was inspired by the proposed mechanism of the incorporation of free cholesterol into nascent VLDL [64], that is, the spontaneous transfer from membranes to lipoproteins [65]. The hypothesis involves also the α-TTP-mediated trafficking of vitamin E from late endosomes (where vitamin E occurs after cellular uptake and large parts of α-TTP are located [66]) to the plasma membrane. This process might involve ABCA1, which has been shown to transport α-TOH [67] and could thus present vitamin E to α-TTP at the outer leaflet of the endosomal membrane. After the transport to the plasma membrane, a yet unidentified flippase is required to transfer α-TOH to the appropriate site of the membrane for uptake by nascent VLDL [30]. This hypothesis is supported by findings of Chung et al. [33], which provided a model of α-TTP-facilitated trafficking of vitamin E from endosomes to the plasma membrane (the reader is referred to Section 2.2 "Intracellular trafficking of vitamin E"). Taken together, the release of α-TOH from hepatocytes depends on vesicular transport [21, 31, 63, 68, 69], but is independent from ER or Golgi [63]. Hence, lipoproteins are not loaded with TOH during their intracellular assembly, but rather after exocytosis, a mechanism is required for the presentation of α-TOH at the plasma membrane. Evidence has been provided that the trafficking of α-TOH to the plasma membrane is realized via α-TTP which is located at recycling endosomes in hepatocytes [33]. However, the mechanism of

the loading of lipoproteins with α-TOH from the plasma membrane has not been elucidated yet, although the involvement of ABC transporters has been suggested [56, 67, 70]. However, ABC transporters are fueling HDL particles, which is in contrast to the assumption that the hepatic release of α-TOH is mediated via VLDL. In turn, two explanations have evolved: first, α-TOH translocates spontaneously from the membrane to VLDL like free cholesterol [65], and second, α-TOH is transported to HDL via ABCA1 and is then spontaneously transferred to VLDL [71]. However, both hypotheses need evaluation. A recent report on the self-assembly of α-TTP to form nanoparticles and transport vitamin E to tissues protected by endothelial barriers like the brain [34] opens another possible way for the distribution of vitamin E throughout the body starting from the liver.

3. Factors influencing hepatic handling of vitamin E

3.1. Effects of vitamin E

3.1.1. Intracellular handling of vitamin E

Key factors in the hepatic handling of vitamin E have been outlined in the previous sections. This section will focus on the action of vitamin E on its own intracellular handling. As indicated above, the key enzyme for the intracellular trafficking of vitamin E is α-TTP, and the rate-limiting enzymes in vitamin E metabolism are CYP4F2 and CYP3A4. Hence, we will here focus on the known actions of vitamin E on these key players.

The key protein of the hepatic handling of vitamin E is α-TTP, with its implications in cellular trafficking, metabolism, and release of vitamin E. Hence, several studies have been conducted to elucidate a possible feedback regulation of α-TTP in response to vitamin E intake, resulting in alterations of the metabolism or the distribution of the vitamin. In principle, research is focused on three levels of regulation: mRNA expression, protein expression, and stabilization of α-TTP protein. However, contradictory results from rodent models have been reported. Fechner et al. found that hepatic α-TTP mRNA expression was strongly induced in rats depleted from vitamin E for 5 weeks after the intake of a TOH-supplemented diet for 24 h [72]. However, rats fed a vitamin E-depleted diet, control diet, or vitamin E-enriched diet for 20 weeks showed upregulation of α-TTP mRNA when vitamin E is deprived, but a downregulation when vitamin E was repleted. Hepatic α-TTP protein levels were comparable for depletion and control, but lowest in rats fed the repleted diet [73]. A similar study reported no differences in hepatic α-TTP mRNA levels of rats fed either a control diet or a diet rich in or low in vitamin E. However, in contrast to the aforementioned study, downregulation of α-TTP protein was reported in the vitamin E-depleted group, while high vitamin E intake did not alter the levels compared to control [74]. The lack of an effect of a vitamin E deficient diet for 290 days on hepatic α-TTP mRNA levels was also reported in another rat model [75]. In line with this, subcutaneous injection of vitamin E for up to 18 days did not alter α-TTP protein levels in rats [76]. However, mice fed a diet rich in vitamin E showed 20% higher hepatic α-TTP protein levels than mice fed a low vitamin E diet [77]. Taken together, some studies

report elevated α-TTP levels due to a higher intake of vitamin E [72, 77], but some revealed no effect [74–76] or even lower levels [73]. Hence, further studies are needed to clarify the role of vitamin E in the regulation of α-TTP. In addition, an *in vitro* study suggested that vitamin E does not regulate α-TTP at the level of gene expression, but stabilizes α-TTP at the protein level upon binding and thus protects the protein from degradation, leading to higher α-TTP protein levels [78]. Reports on the hepatic mRNA levels might thus be of minor importance for the interpretation of the contribution of vitamin E to α-TTP action; however, the findings on α-TTP protein expression are also inconsistent.

The rate-limiting enzymes of vitamin E metabolism are CYP4F2 and CYP3A4. The latter was reported to be under transcriptional control of pregnane-X-receptor (PXR) [79, 80]. Hence, vitamin E might regulate its metabolism by binding to PXR and subsequent alteration of the expression of the enzymes involved in the first catabolic step. Indeed, studies using cells transfected with reporter genes provided evidence for an activation of PXR by different vitamin E structures (i.e., TOHs, T3s, and metabolites) [81, 82]. Interestingly, α-, δ-, and γ-TOH as well as α- and γ-T3 activated PXR in HepG2 liver cells transfected with human PXR and chloramphenicol acetyl transferase linked to two PXR responsive elements [81], while α- and γ-TOH as well as their metabolites α- and γ-CEHC did not in transfected colon carcinoma cells [82]. However, the LCM α-13′-COOH activated PXR in the latter cellular system and so did γ-T3 [82]. This finding implicates that the LCM of TOH are the responsible mediators of reported TOH actions via PXR. Hence, the findings in hepatic HepG2 cells [81] might be due to a higher catabolic rate of TOH and in turn the more efficient formation of the LCM than in colon cells. However, these findings were made in artificial cellular reporter systems and might not resemble the actual (hepatic) situation *in vivo*. Further, the specificity of PXR might depend on the species, as γ-T3 (the vitamin E form that activated PXR in both of the aforementioned studies) fails to bind murine PXR [83]. However, results obtained *in vivo* support the regulation of Cyp3a11 (the murine orthologue of CYP3A4) by vitamin E via PXR. Mice supplemented with α-TOH show elevated hepatic expression of Cyp3a11, while their PXR-deficient counterparts as well as mice with humanized PXR showed no upregulation of Cyp3a11 in response to α-TOH [84]. The same finding was made for Cyp4f13, the murine orthologue of CYP4F2, in this model [84]. These findings suggest that both enzymes are under the control of PXR and murine, but not human PXR is susceptible to α-TOH (or its metabolites as outlined above). Further studies reporting upregulation of hepatic Cyp3a in rodent models with α-TOH supplementation support this finding [76, 83, 85]. Interestingly, in these studies, γ-TOH and γ-T3 had no effect on Cyp3a expression [83, 85], supporting the suggested specificity of murine PXR for α-TOH. In line with this, γ-TOH did not alter the expression of Cyp4f13 in mice [85]. However, subcutaneous application of α-TOH in rats did not induce Cyp4F2 levels [76], which is in contrast to the above mentioned induction of Cyp4f13 in mice via PXR [84]. The reported induction of CYP4F2 activity in HepG2 cells by α-TOH further complicates the interpretation of the data on the effect of vitamin E on CYP4F2 [45]. Taken together, there is evidence for the regulation of CYP4F2 and CYP3A4 via PXR by vitamin E in the human liver. However, several aspects need further clarification, for instance, species and vitamin E isoform specificity of PXR, the regulation of CYP4F2 by vitamin E or the relevance of the α-LCM as true mediators of α-TOH effects via PXR.

3.1.2. Vitamin E intake

Several key enzymes determine the rate of vitamin E catabolism (the reader referred to Section 2.4 "Hepatic metabolism of vitamin E") and, as outlined in the previous section, there is evidence that vitamin E in general might regulate its own metabolism. However, there are differences in the ability to regulate the metabolism depending on structural properties of the vitamin E isomers (i.e., methylation of the chroman ring, saturation of the side-chain, and stereochemistry). In principle, high intake of vitamin E, independent from the isomer, leads to enhanced formation of the respective metabolites [49]. However, the catabolic rates of the different forms of vitamin E clearly differ: the γ-isoforms are more susceptible to metabolization than the α-isoforms. Subjects supplemented with γ-T3 and α-T3 (125 mg or 500 mg) showed four to six times higher urinary excretion of the catabolic end product γ-CEHC and an induction of α-CEHC only after high dose (500 mg), but not after low dose supplementation (125 mg) [86]. In line with this, equimolar supplementation with 50 mg of α- and γ-TOH leads to a twofold increase of plasma γ-CEHC, but no alterations in α-CEHC [87]. These data indicate that there might be a threshold for the intake of α-TOH and α-T3 (or plasma levels, respectively) that needs to be exceeded to accelerate catabolism of α-TOH and α-T3 to form α-CEHC, as suggested by Schuelke et al. [88]. Interestingly, already in 1985, Handelman et al. reported that high α-TOH levels in human plasma are related to low γ-TOH levels [89]. After supplementation of α-TOH, the plasma α-TOH levels were, as expected, twofold to fourfold higher, but the γ-TOH level decreased to between one-third and one-half of the initial level [89]. Hence, α-TOH intake seems to boost γ-TOH catabolism. Supporting data were generated in a rat model, where the combined supplementation of α- and γ-TOH leads to higher excretion of γ-CEHC than the supplementation of γ-TOH alone [90], as well as the reported stimulation of γ-TOH catabolism by α-TOH in HepG2 liver cells [91]. Although the underlying mechanisms are not fully unraveled, there is evidence that α-TOH induces the activity of enzymes involved in the metabolism of vitamin E, leading to the degradation of non-α-forms, while α-TOH remains protected (please refer to Section 3.1.1 "Intracellular handling of vitamin E").

3.2. Effects of other compounds

3.2.1. Intake of sesamin

Sesamin is a lignan, a group of natural compounds derived from vegetable sources, like sesame seeds [92]. Sesamin is known as a natural inhibitor of the metabolism of TOH [93–97]. The cell regulatory actions of sesamin have been initially investigated in *in vitro* models, where Parker et al. showed that sesamin acts as a selective inhibitor of CYP3A4, an initial enzyme of TOH metabolism [46]. In this study, the authors compared the inhibitory potential of sesamin on TOH metabolism in human HepG2 cells to the well-characterized CYP3A4 inhibitor ketoconazole. HepG2 cells were treated with one of the mentioned compounds in combination with either 25 μM α-TOH or 25 μM γ-TOH. Afterwards, the concentration of the corresponding CEHC was determined as a marker for TOH metabolism in cell culture media. It became apparent that ketoconazole (1 μM) and sesamin (1 μM) inhibited the formation of α- and γ-CEHC. This result provides evidence that sesamin is able to modulate TOH metabolism via the inhibition of

CYP3A4 [46]. In addition to the *in vitro* data, Uchida and coworkers investigated the inhibitory effects of sesamin on vitamin E metabolism in rats. Vitamin E-deficient rats (vitamin E free diet for 4 weeks) were treated with 50 mg/kg *RRR*-α-TOH alone or in combination with 200 g/kg sesame seeds [95]. Next, the concentration of α-TOH in different tissues as well as the urinary excretion of α-CEHC was measured. The urinary excretion of α-CEHC in the sesamin group was significantly lower compared to the α-TOH control group. Further, the combination of α-TOH and sesamin provoked a significant increase of hepatic α-TOH concentrations compared to α-TOH treated animals [95]. These observations have been confirmed in other animal studies [93, 94]. Beside the investigations in animal models, there are also a few results originating from studies in humans. In 2004, Frank and colleagues used muffins enriched with sesame oil (94 mg sesamin/muffin) or corn oil (control) to investigate the effect of a single dose sesamin application on urinary excretion of γ-CEHC as well as blood levels of γ-TOH in 10 healthy volunteers [97]. Both, control and intervention group, received the muffins together with a capsule containing deuterium-labeled γ-TOH (50 mg) in a crossover design. Blood and urine samples were collected over 72 hours after the application of the muffins and capsules. While the urinary excretion of γ-CEHC was significantly lowered, the sesamin treatment did not affect γ-TOH concentrations in blood compared to the corn oil control group [97]. Unfortunately, the study does not provide data on the elevation of the hepatic γ-TOH concentration in response to the reduced urinary excretion of γ-CEHC. Taken together, *in vitro* and *in vivo* studies provide evidence that the dietary intake of sesamin leads to an increase of the hepatic concentration of TOH via the inhibition of vitamin E metabolism, but further experiments are needed to characterize the interaction of sesamin and vitamin E metabolism in more detail.

3.2.2. Pharmacological activation or inhibition of CYP3A4

The pharmacological modification of the enzymatic activity of CYP3A4 represents an effective way to influence vitamin E homeostasis in the human body. Mechanistically, the direct or indirect interference of vitamin E metabolism is usually just a side effect of the pharmacological inhibition or induction of CYP3A4 by various chemical compounds. Thus, it is not surprising that the first evidence for the involvement of CYP3A4 in vitamin E metabolism was provided in an experimental subset using ketoconazole as a specific inhibitor for CYP3A4 [46, 98]. In HepG2 liver cells, different concentrations of ketoconazole (1 mmol/l or 0.25 mmol/l) inhibited the metabolic conversion of γ- and δ-TOH (25 μmol/l cell culture media) to γ- or δ-CEHC by almost 90% [46]. This finding has been confirmed by the reproduction of the same experiment with sesamin, the natural inhibitor of CYP3A4, revealing comparable results [46]. The inhibitory effect of ketoconazole on vitamin E metabolism has further been observed in an *in vivo* model. Here, rats were supplemented with ketoconazole (50 mg/kg body weight) together with α-TOH (10 mg/kg body weight), γ-TOH (10 mg/kg body weight) or mixture of different T3s (29.5 mg/kg body weight). Ketoconazole significantly reduced the catabolism of all applied vitamin E forms resulting in impaired urinary excretion of the respective CEHCs [99]. Beside its inhibition, the pharmacological induction of CYP3A4 represents another way to modulate vitamin E metabolism. Birringer and coworkers demonstrated that 50 μmol/L rifampicin, an inducer of CYP3A4 activity [100], induced the degradation of all-*rac*-α-TOH

in HepG2 cells fivefold [47]. In this study, the cell culture medium has been preconditioned with 100 μmol/L α-TOH for 10 days, as the standard medium was deficient for α-TOH [47]. Further, an indirect approach for the modulation of vitamin E metabolism via the modification of CYP3A4 expression could be realized by triggering PXR, a nuclear receptor that regulates the expression of metabolic enzymes and transporters involved in the metabolism of xenobiotics and endobiotics [101, 102]. Landes and coworkers showed that γ-T3 as well as rifampicin acts as PXR agonists, thus upregulating CYP3A4 mRNA expression in HepG2 liver cells [81]. Given the fact that enhanced mRNA expression of CYP3A4 results in enhanced enzymatic activity, the stimulation of PXR by various pharmacological agonists or antagonists could also modulate the hepatic metabolism of vitamin E. In summary, the direct or indirect regulation of CYP3A4 by various pharmacological means represents an effective way to modify the hepatic vitamin E metabolism.

3.3. Nonmodifiable factors influencing handling of vitamin E

The handling of vitamin E is also influenced by nonmodifiable factors. These are aging, gender, and individual genetics. Published data in this area are sparse but interesting.

3.3.1. Aging

The aging process is characterized by nine hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [103]. In particular, the mitochondrial dysfunction leads to higher formation of reactive oxygen species (ROS) and enhanced oxidative damage [104]. Both processes can be diminished by the antioxidant function of vitamin E [105]. Consequently, two questions arise: (i) can vitamin E modulate the aging processes or prevent age-related diseases? This has been subject of several reviews [106–109]. (ii) And how is the concentration, distribution, and function of vitamin E modulated by the aging process? In humans, age-dependent changes of α-TOH plasma concentrations are known. In healthy aged humans, the α-TOH plasma concentrations are higher than in younger individuals [110–113]. However, this might be due to the age-related increase of plasma cholesterol concentrations, as the age-related increase in α-TOH plasma concentrations disappear after adjustment for cholesterol plasma concentrations [112] or serum lipids [113]. Traber et al. suggested that α-TOH plasma concentrations are more dependent on control mechanisms for plasma lipids rather than on α-TOH absorption [113]. Hospitalized elderly patients [114] as well as older persons with cognitive impairments (dementia or Alzheimer's disease [115, 116]) have low α-TOH plasma concentrations [117]. However, an unfavorable nutrient status of the hospitalized patients was discussed as the cause of the lower α-TOH plasma concentrations.

Several studies analyzed the age-dependent changes of α-TOH tissue concentrations and handling in mice [37, 117–119] and rats [120]. In brain [37, 117, 118] and kidney [37, 117], epididymal adipose tissue [117] and aortic vessel wall [120], a consistent increase in α-TOH was found with age. In old rats, however, an age-dependent increase in intestinal absorption was found [121]. This was considered as a *"self-protective age-dependent adaption"* [120], which

is thought to counteract increased oxidative stress during aging. In the liver and heart, however, data are conflicting: while some found increased concentrations [37, 119, 120], Takahashi et al. found decreased values [117]. Two studies also analyzed the age-dependent regulation of genes, known to be involved in vitamin E handling, which are α-TTP, ABCA1, and Cyp4f14 (murine orthologue of CYP4F2) [117] as well as NPC1, NPC2, and LPL [37]. Takahashi et al. found increasing (mice with the age of 3–12 month) and then decreasing (12—24 months) α-TTP protein levels in the liver, while mRNA expression was stable over age [117]. Overall, Cyp4f14 mRNA expression decreased during aging (60% decrease in mRNA expression at the age of 24 months compared to the age of 3 weeks), while ABCA1 mRNA expression slightly increased (20% in the same age range as measured for Cyp4f14) [117]. The authors concluded that the age-related changes of hepatic α-TOH levels cannot be explained by the metabolism of α-TOH via Cyp4f14. König et al. analyzed protein expression in kidney tissue or its lysosomal membranes and found a significant decrease of NPC1 and NPC2, but a prominent increase in LPL (361% compared with the tissue from younger mice) [37]. The increased expression of LPL may explain the accumulation of α-TOH in aged mice. Furthermore, NPC1 and NPC2 may be responsible for the transport of α-TOH from the endosomes to the cytosol [69] and their reduced expression may explain the accumulation of α-TOH in lysosomal membranes [37]. In summary, there are age-dependent changes in α-TOH tissue and plasma concentrations and also in the expression of genes responsible for vitamin E handling; however, the underlying regulatory processes are not unraveled completely yet.

3.3.2. Gender

The sex-dependent differences in vitamin E handling were described recently by Schmölz et al. [6] and will be summarized here briefly for humans only. While intake of vitamin E in total is higher in men than in women [122], the intake per kcal is higher for women than for men [123]. The absorption of α-TOH seems not to be influenced by sex, but is mainly regulated by downstream regulatory processes (likely by hepatic sorting or metabolism) [113]. The data on serum concentrations of vitamin E are inconsistent: while some researchers reported elevated α-TOH serum concentrations for women compared to men [124, 125], others found contradictory results [123]. Sex-dependent regulation of vitamin E metabolism is specific for the different forms of vitamin E. Women degrade γ-TOH to a higher degree than men, while the metabolism of α-TOH seems to be independent [87]. Two mechanisms may be relevant for sex-dependent regulation of vitamin E metabolism: the hormonal status of individuals and the activation of the CYP enzymes involved in vitamin E metabolism [6]. Further studies could illuminate gender-specific differences in more detail. In the light of the discovery of vitamin E as a factor that limits female fertility, this is of special interest.

3.3.3. Genetics

The influence of genetics on vitamin E handling was summarized in detail in a recent review (for more details, please see [6]). Therefore, only a short overview will be provided here. Interindividual differences in the handling of vitamin E can be caused by individual genetic constitutions. Polymorphisms in genes, which are responsible for vitamin E handling such as

CYP4F2 [126], NPC1L1 [127], and CD36 [128] are likely to contribute to variations in vitamin E status. The best-studied gene in this context is α-TTP, as its genetic variability may cause AVED. Two genetic variants are known, which are located in or nearby the proposed tocopherol-binding domain and cause reduced α-TOH serum concentrations [129]. Furthermore, mutations in the promoter region of α-TTP (with increased or decreased activity) were also reported [130]. In summary, vitamin E handling is influenced by several mechanisms, one of which is the variability of genes involved in these processes. This might held responsible for interindividual differences in vitamin E serum concentrations.

3.4. Pathophysiological factors influencing handling of vitamin E

3.4.1. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis

Nonalcoholic fatty liver disease encompasses a histological spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is a clinical symptom characterized by a pattern of steatosis, inflammation, and hepatocyte ballooning, which can result in the development of cirrhosis and liver cancer [131]. Although the molecular mechanisms of NASH development remain poorly understood, studies provide evidence for a critical role of oxidative stress together with an impaired antioxidative response [132, 133]. In line with this, Erhardt and coworkers observed significantly lower plasma levels of α-TOH and other antioxidants in NASH patients compared to healthy controls [134]. Given the fact that an induction of CYP3A4 or CYP4F2 results in decreased vitamin E concentrations in the human body, it has been expected that NASH leads to an enhanced activity or expression of these enzymes. Thus, Woolsey and coworkers investigated the enzymatic activity as well as the mRNA expression of CYP3A4 in NASH patients [135]. The authors used liver biopsies for mRNA analyses and determined the concentration of 4β-hydroxycholesterol in plasma as an endogenous biomarker for CYP3A4 activity. Interestingly, NASH patients showed a 37% reduced enzymatic activity of CYP3A4 as well as a 69% lower CYP3A4 mRNA expression compared to healthy controls [135]. Unfortunately, there is no further data on the activity or the expression of CYP4F2 in NASH patients. However, Athinarayanan and coworkers investigated the influence of two different CYP4F2 genotypes (V433 M and W12G) on vitamin E plasma concentrations in NASH patients [136–138]. The V433 M genotype was associated to higher baseline levels of vitamin E, indicating lower enzymatic activity compared to the W12G genotype [136–138]. Thus, the authors hypothesized that the W12G genotype in NASH patients could explain the lower vitamin E plasma concentrations. However, this hypothesis has been disproved by the finding that the vitamin E plasma concentrations of NASH patients did not differ between the two CYP4F2 genotypes [136–138]. Based on the available data, CYP4F2 and CYP3A4 seem to have no influence on vitamin E plasma concentrations during the NASH development. Next to the CYPs, α-TTP could also be involved in a potential mechanism explaining the observation of Erhardt and coworkers mentioned above. In line with this, Ban and coworkers used a rat model to investigate whether an exposure to hyperoxia (>95% O_2 for 48 h), an established stimulus for ROS production [139], could alter the expression of hepatic α-TTP [140]. Indeed, hyperoxia decreased the expression of α-TTP mRNA in rat liver, while α-TTP protein expression remained unchanged [140]. As oxidative stress and ROS

formation are crucial factors for NASH development, lowering α-TTP expression by ROS could explain the lower vitamin E levels in NASH patients. In summary, the concentration of vitamin E and other antioxidants is reduced in NASH patients by yet not fully understood molecular mechanisms, potentially involving α-TTP. Nevertheless, recent human intervention trials provide evidence that vitamin E treatment could improve primary NASH outcomes (i.e., steatosis, inflammation, hepatocellular ballooning, and fibrosis) [137, 138].

3.4.2. Cancer

The current data on vitamin E as a potential agent for cancer therapy are inconsistent. While *in vitro* and early epidemiological studies provided evidence for cell growth-inhibiting, antiproliferative and pro-apoptotic effects of vitamin E in cancer treatment [141–145], more recent investigations reported contradictory results [146–148]. These findings were further sustained by the "Selenium and Vitamin E Cancer Prevention Trial (SELECT)," a randomized intervention study to determine the long-term effect of a supplementation of vitamin E (400 IU/d all-*rac*-α-tocopheryl-acetate) and selenium (200 μg/d L-selenomethionine) on the risk of prostate cancer in healthy men. Interestingly, the authors observed an increased incidence for prostate cancer in subjects supplemented with vitamin E [149]. Beside the investigations on beneficial effects of vitamin E in cancer therapy, almost nothing is known about the influence of cancer on human vitamin E homeostasis. An early study by Knekt, who investigated the association of vitamin E serum concentrations and the risk for different types of female cancer, showed an inverse relation between α-TOH serum concentrations and cancer risk [150]. Thus, women with the lowest α-TOH levels were at enhanced risk for cancer compared to those with higher α-TOH levels. Indeed, this association was restricted to cancer outcomes in tissues and organs, which were not exposed to estrogens [150]. Thus, Knekt hypothesized that low vitamin E levels could represent a potential risk factor for several, but not all types of cancer [150]. Nevertheless, the molecular mechanisms underlying this impairment of vitamin E serum concentrations in cancer patients remain unclear. The enhanced metabolic conversion of vitamin E might represent a mechanistic explanation. In line with this, investigations of tissues from cancer patients showed elevated expression of CYP3A4 [151] and CYP4F2 [152], the two major enzymes of vitamin E catabolism. Unfortunately, vitamin E serum concentrations have not been determined in these studies. Further, *in vitro* studies provided evidence that cancer also affects transporters for vitamin E, such as the tocopherol-associated protein (TAP) [153]. Tissue samples from prostate cancer patients showed significantly lower TAP mRNA expression compared to healthy controls, indicating that cancer may affect the intracellular transport of vitamin E. In addition, the overexpression of TAP in prostate cancer cells leads to a significant reduction of cell growth, while a TAP knockdown by small interfering RNA increased their growth [153]. Interestingly, these effects appeared without additional vitamin E treatment, indicating that TAP not only mediates vitamin E transport but also functions as a vitamin E-independent tumor suppressor gene [153]. In summary, the promising cancer preventive effects of vitamin E shown *in vitro* have not been confirmed in recent *in vivo* trials. Nevertheless, cancer could probably be associated with reduced vitamin E concentrations in the human body, because of an enhanced vitamin E catabolism and/or the alteration of its intracellular transport. However, further investigations are required to validate these results.

3.4.3. Disorders of lipoprotein metabolism

After its intestinal absorption, the transport of vitamin E, including its transfer to and its export from the liver as well as the subsequent distribution of vitamin E in the human body, strictly depends on different lipoproteins [7]. Thus, disorders of the lipoprotein metabolism can lead to disturbances of vitamin E homeostasis. Abetalipoproteinemia or Bassen-Kornzweig syndrome is a rare form of neurodegenerative ataxia with a strong impact on the hepatic handling of vitamin E. Abetalipoproteinemia is caused by mutations in the gene encoding for the microsomal triglyceride transfer protein (MTP), which is required for the assembly and secretion of the apolipoprotein B (apoB) forms in the liver and the intestine [154]. The apoB forms are the primary apolipoproteins associated to chylomicrons or VLDL, IDL, and LDL, respectively, and are thus essential for the distribution of vitamin E in the human body [7, 155]. As a result of the disturbed intestinal absorption and hepatic excretion of all lipid soluble molecules, patients with abetalipoproteinemia show vitamin E deficiency as well as low serum concentrations of cholesterol and triglycerides [156]. Next, the hepatic handling of vitamin E can be affected by familial hypobetalipoproteinemia. This lipoprotein disorder is caused by mutations in the *APOB* gene, leading to disturbances of translation of the apoB proteins and/or impaired secretion of VLDL [157]. Thus, familial hypobetalipoproteinemia displays the same clinical features as abetalipoproteinemia. In summary, lipoprotein disorders exert clear impact on the hepatic and systemic handling of vitamin E.

3.4.4. Other relevant pathophysiological factors

AVED is a neurological disorder, which has for the first time been described in a 12-year-old boy with cerebellar ataxia and low serum vitamin E concentrations. Interestingly, the boy showed no lipid malabsorption or a lack of lipoproteins, like it has been observed in abetalipoproteinemia [158]. Subsequent studies identified a mutation in the *TTPA* gene, the gene encoding for α-TTP, as the disease causing factor [159]. Thus, AVED patients have impaired expression of α-TTP, leading to impaired incorporation of vitamin E (α-TOH) into VLDL as well as a higher metabolic conversion and excretion of vitamin E [154]. In addition, AVED patients show very low plasma vitamin E concentrations together with normal absorption rates for vitamin E in the absence of intestinal malabsorption and abetalipoproteinemia [2, 154]. In summary, AVED represents a clinical condition that includes altered hepatic handling of vitamin E without affecting lipoprotein homeostasis.

4. Conclusion

In the last decades of vitamin E research, the liver appeared as the central organ for the uptake, distribution, metabolism, and storage of vitamin E. Thus, it is also a starting point for various strategies for the modulation of the vitamin E homeostasis. Based on current knowledge, we identified physiological, nonphysiological as well as pathophysiological factors influencing the hepatic handling of vitamin E, verifying the crucial role of the liver in vitamin E homeostasis (a brief schematic overview is provided in **Figure 1**). Nevertheless, further studies

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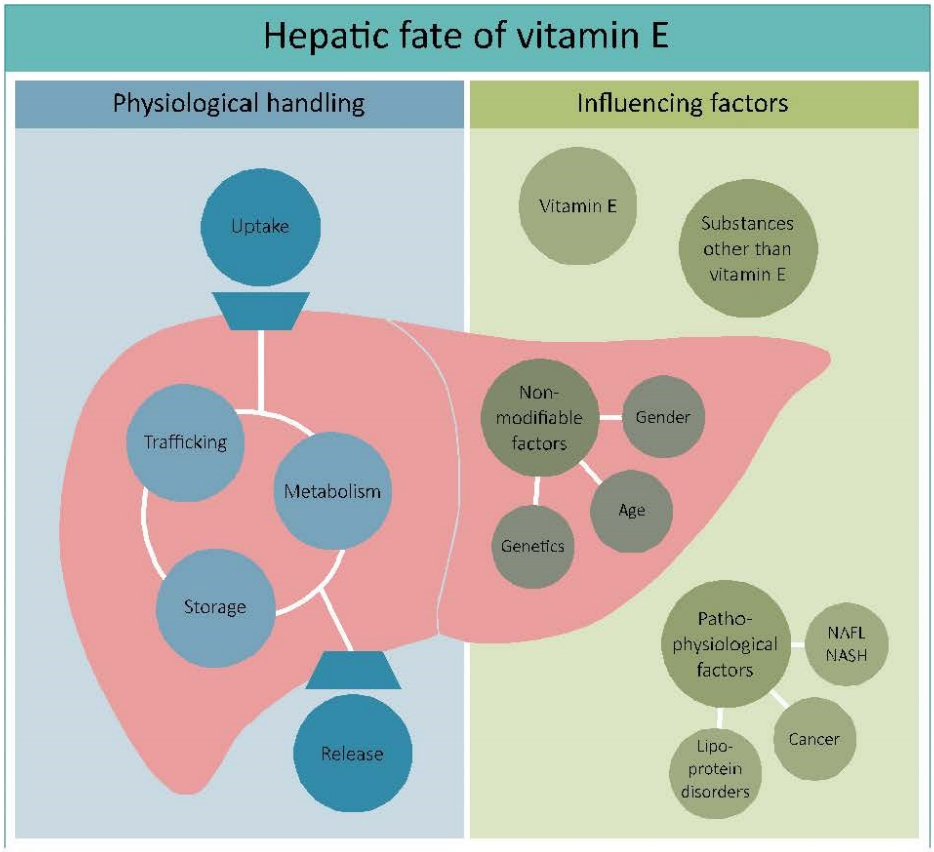

Figure 1. The crucial role of the liver in vitamin E homeostasis.

are needed to unravel the molecular mechanisms underlying the described disturbances of hepatic vitamin E handling by various factors.

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7.9 Manuscript IX

Chapter 6
Bioactivity of Vitamin E Long-Chain Metabolites

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Keywords Vitamin E · Long-chain metabolites of vitamin E · 13′-hydroxychromanol (13′-OH) 13′-carboxychromanol (13′-COOH) · Vitamin E metabolism · Biological activity

Key Points

- Metabolic activation of vitamin E precursors by hepatic catabolism
- Unknown distribution mechanisms and storage of LCMs (i.e., occurrence in organs and tissues)
- Detection of both α-LCMs in complex biological matrices
- Involvement of the LCMs in various regulatory processes
- Evidence for a general concept of metabolic activation for fat-soluble vitamins

Abbreviations

Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
APCI	Atmospheric pressure chemical ionization
α-TTP	α-Tocopherol transfer protein
ABCA1	ATP-binding cassette transporter A1
13′-COOH	Carboxychromanol

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CEHC	Carboxyethylhydroxychromanol
CD36	Cluster of differentiation 36
CYP	Cytochrome P450
ECD	Electrochemical detector
ESI	Electrospray ionization
FID	Flame ionization detector
FLD	Fluorescence detector
GC	Gas chromatography
HPLC	High-pressure liquid chromatography
13′-OH	Hydroxychromanol
ICM	Intermediate-chain metabolite
LCM	Long-chain metabolite
LDL	Low-density lipoprotein
LDLR	LDL receptor
LRP	LDL receptor-related protein
MS	Mass spectrometry
LC3	Microtubule-associated protein 1 light chain 3
PBMC	Peripheral blood mononuclear cell
P-gp	P-Glycoprotein
PARP	Poly-ADP ribose polymerase
Q-TOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
SR-B1	Scavenger receptor B1
SCM	Short-chain metabolite
SPE	Solid phase extraction
TOH	Tocopherol
T3	Tocotrienol
UV-Vis	Ultraviolet visible spectroscopy
VLDL	Very low-density lipoprotein

Formation of Vitamin E Long-Chain Metabolites

The metabolism of vitamin E is initiated by an ω-hydroxylation via cytochrome P450 (CYP) 4F2/3A4, resulting in the formation of 13′-hydroxychromanols (13′-OH). The subsequent ω-oxidation forms 13′-carboxychromanols (13′-COOH). Subsequent cycles of β-oxidation shorten the side chain and finally result in water-soluble carboxyethylhydroxychromanols (CEHCs or 3′-COOH). A more detailed description is here provided in the chapter "Vitamin E metabolism." The knowledge about the regulatory processes of vitamin E metabolism is sparse, since the responsible enzymes are largely unknown (except CYP4F2 and CYP3A4) or have not yet been experimentally confirmed. Torquato et al. provided first hints by the observation of the upregulation of CYP4F2 protein by α-13′-OH in human HepG2 liver cells, indicating a positive regulatory feedback loop [1]. Whether this holds true for the other long-chain metabolite (LCM) forms, i.e., β-, γ-, or δ-13′-OH, or even for other metabolites (e.g., α-13′-COOH), is subject of further investigations. The enzymes that are responsible for the ω- and β-oxidation have not yet been experimentally validated, but Mustacich et al. described the organelles that are responsible for the metabolism of vitamin E and suggested the respective enzyme classes [2]. Based on this work, the identification of the aldehyde and alcohol dehydrogenases that are responsible for the ω-oxidation of 13′-OH is possible. Furthermore, the enzymes that are responsible for the degradation of branched-chain fatty acids have been suggested to degrade also the side chain

of vitamin E. However, this also requires validation in future studies. The successful identification of the respective enzymes is a vital starting point for unraveling regulatory mechanisms of vitamin E metabolism.

Distribution of Vitamin E Long-Chain Metabolites in the Human Body

Hepatic Formation and Transport of Vitamin E Long-Chain Metabolites

Storage and metabolism of vitamin E are strictly balanced in healthy humans to ensure constant and sufficient supply. Therefore, hepatic metabolism of vitamin E is regulated by a physiological feedback loop to avoid excessive accumulation of vitamin E and to bridge periods of vitamin E deficiency. All ingested vitamin E forms are first transferred via chylomicrons to the liver, but α-tocopherol (TOH) is selectively bound by the α-tocopherol transfer protein (α-TTP) [3] to direct it either for release or metabolism in different cellular compartments, the endoplasmic reticulum, the peroxisomes, and the mitochondria (for details, the reader is referred to the chapter "Vitamin E metabolism"). Hence, the intermediate metabolites of vitamin E must be transported between these compartments during their metabolic degradation. But, no experimental data is available whether this inter-compartment transport occurs by passive diffusion or is conducted by active transport via specific proteins. However, non-metabolized vitamin E is released from the liver, and the contribution of very low-density lipoproteins (VLDL) [4], oxysterol-binding proteins [5], and the ATP-binding cassette transporter A1 (ABCA1) [6] to this process has been discussed. The chemical structure of the α-13′-long-chain metabolites (α-13′-LCMs) and α-TOH is similar, except for the terminal oxidation of the aliphatic side chain. Therefore, the lipophilic nature of these molecules may be similar, and their transport via VLDL is conceivable but needs to be verified in future studies. In addition, the intracellular transport of the LCMs may occur via specific LCM-binding and LCM-transport proteins or via binding and transport proteins jointly used for α-TOH (e.g., α-TTP [3], tocopherol-associated protein [7]) or fatty acids (e.g., fatty acid-binding proteins [8]), since α-TOH follows the general transport pathway of lipids [9]. However, whether the transport of the LCMs is realized by specific (i.e., binding and transport proteins) or unspecific (i.e., via lipoproteins) mechanisms remains unclear.

Extrahepatic Transport and Storage

Only limited information about the transport of the LCMs in blood and their bioavailability in extrahepatic tissues is yet available. α-13′-OH [10] and α-13′-COOH [11] have been found in human serum, and increased serum concentrations after supplementation with 1000 IU α-TOH/d were observed [10, 11]. It might be possible that their extrahepatic transport in blood occurs via lipoproteins as it has been described for α-TOH [12]. Vitamin E in general is packed into chylomicrons after intestinal absorption and later transferred to other lipoproteins like HDL via phospholipid transfer protein, or it remains in chylomicron remnants. After the hepatic uptake, discrimination for α-TOH and resecretion into the blood via VLDL take place. According to the known fate of VLDL, α-TOH also occurs in LDL and HDL particles [13]. However, further studies are needed to clarify the involvement of lipoproteins or specific binding proteins in the distribution of the LCMs. The cholesteryl ester transfer protein, another member of the protein family of serum lipid transfer proteins, is also discussed to contribute to vitamin E transport and metabolism [14] and may also be involved in the transport of the LCMs. Until today, no specific plasma transport proteins for α-TOH [12, 15] nor the LCMs have been described.

It has been shown that normal vitamin E plasma levels of 25 μM can be increased about threefold by vitamin E supplementation, whereas α-13′-COOH serum levels increased even about eightfold. Preliminary studies in mice showed that α-13′-COOH reaches its highest concentrations in plasma 6 h after injection (unpublished data). Based on the increase of the LCMs after supplementation and the fast rise of plasma concentration and clearance from blood, LCMs might reflect the current nutritional status with respect to vitamin E. However, this hypothesis needs confirmation under non-supplemented normal conditions. As adipose tissue and skeleton muscles have been identified as long-term storage depots for vitamin E and the LCMs exhibit structural similarities to their respective vitamin E precursors, similar storage characteristics are conceivable [16, 17]. Hence, continuous release of the LCMs from these depots is likely. Nevertheless, a reliable analysis of tissue distribution and tissue-specific concentration of the LCMs is needed to draw conclusions about their bioavailability, stability, overall physiological relevance, and relevance in single organs. Thereby, the potential of these molecules for the therapeutic treatment of organ-related diseases such as nonalcoholic fatty liver disease should be studied. Next, investigations of putative regulatory feedback loops for the uptake of the LCMs are needed. Beside the major storage organs and tissues known for α-TOH (i.e., fat and muscles), the accumulation of α-TOH and its corresponding metabolites has also been shown for the kidney and small intestine [18]. Further, the analysis of the concentrations of the LCMs in the brain, since vitamin E deficiency is known to cause cognitive dysfunctions [19], as well as the heart, where the concentration of α-TOH increases after supplementation [20], is of interest. In addition, α-TTP is expressed in the placenta, indicating the importance of α-TOH for preventing fetus resorption [15]. However, to date there is no data available on the contribution of α-TTP to the tissue distribution of the LCMs. Crucial for the binding specificity of α-TTP is the substitution of the chroman ring system of possible ligands as well as the *R*-configuration at the 2′-position [3]. The phytyl side chain is very flexible, thus barely contributing to the specificity [21]. As the α-LCMs differ only in the terminal modification of the phytyl-like side chain from α-TOH, they may be likely bound by α-TTP with good affinity. Supporting this assumption, α-TOH analogues with terminal side chain modifications, like nitrobenoxadiazyl (NBD)-α-TOH and anthroyloxy (AO)-α-TOH, bind α-TTP and are in use to investigate functions of α-TPP [22, 23]. Hence, terminal modification of the α-TOH side chain does not prevent binding to α-TTP. However, the final confirmation of the binding of the α-LCMs to α-TTP is pending. Apart from human plasma or serum [11, 24], the concentrations of the LCMs in different organs or tissues have not been investigated. Given that only 1% of total body TOH is located in the blood [25], most of the vitamin E is stored in other parts of the body. To date, only serum concentrations of the LCMs are known, and it is possible that other tissues display higher concentrations of the LCMs than the blood.

Intercellular Transport and Intracellular Distribution

In addition to information about the accumulation of the LCMs in organs, investigations of uptake mechanisms for the LCMs into cells and cellular compartments will provide valuable information about intracellular distribution patterns. Initial experiments in human skin fibroblasts, human THP-1 macrophages, and human HepG2 liver cells revealed similar uptake kinetics for the LCMs (unpublished data), but further experiments are needed to characterize the transport mechanisms. As indicated above, the transport via lipoproteins, as known for α-TOH, has not yet been shown for the LCMs. The data on LCM uptake from in vitro experiments indicate for an independent transport mechanism but do not exclude lipoprotein-specific mechanisms. Hence, internalization of particles carrying the LCMs might involve among others the LDL receptor (LDLR) or the LDL receptor-related protein (LRP), as known for α-TOH [13]. In addition, the scavenger receptor B1 (SR-B1) and ABCA1 are of particular importance within the distribution pathways of vitamin E [14]. SR-B1 mediates the uptake of vitamin E into the intestine and in peripheral tissue, whereas LDLR and LRP

mediate the uptake into the liver. The efflux of vitamin E via ABCA1 takes place in the intestine and the liver. In addition, ABCA1 regulates the cellular efflux of vitamin E in macrophages and fibroblasts [6]. Based on its lipophilic nature, vitamin E and thus most likely also the corresponding LCMs are present in the endoplasmic reticulum as well as the peroxisomes [26]. Besides α-TTP, further intracellular binding proteins, such as the tocopherol-associated protein and the tocopherol-binding protein, are known to be involved in intracellular trafficking of α-TOH [27]. Further, the multidrug resistance transporter P-glycoprotein (P-gp) has been identified to mediate α-TOH transport across the plasma membrane and its secretion into bile [28]. All members of this protein family bind α-TOH with lower affinity than α-TTP [13]; however, their relevance as transporters of the LCMs remains unclear. Further experiments are needed to clarify the importance of α-TOH-related binding proteins, such as afamin [29], as well as the uptake mechanisms and kinetics that are responsible for the cellular and tissue distribution of the LCMs and hence their regulatory effectiveness.

Excretion

The metabolism of vitamin E finally forms the hydrophilic end-product α-CEHC, which is excreted via urine or secreted into the circulatory system [18]. In contrast, free α-13′-OH has been found to be excreted with feces [30], as shown earlier for γ-TOH and α-TOH [31]. Therefore, the bioavailability-to-excretion ratio of the LCMs needs to be investigated in further studies. A useful experimental tool to observe and analyze the formation of metabolites in humans is to use deuterium-labeled α-TOH [32]. As shown by Freiser et al., the γ-LCMs are mainly conjugated with sulfates [33] and glucuronides in plasma and are likely excreted. However, biological activity is only possible if the LCMs are available in unconjugated form [34]. To gain deeper insights into the pharmacokinetics of vitamin E, in particular into the formation of the metabolites as well as their bioavailability and distribution in tissues and excretion, robust and reliable analytical tools are indispensable.

Analytical Approaches for Vitamin E Metabolites

In the early days of vitamin E research, simple chromatographic methods were used to separate the different forms of vitamin E from other lipids and lipophilic molecules such as triglycerides and phospholipids. Thin-layer chromatography was one of the early approaches to detect vitamin E in biological samples [35]. Next, the development of gas chromatography (GC) and high-pressure liquid chromatography (HPLC) approaches allowed for better separation of the different vitamers [36, 37]. By coupling of either GC or HPLC to high-sensitive mass spectrometry (MS), vitamin E forms could be detected even in nanomolar concentrations within different matrices [38, 39]. Next, the discovery of vitamin E metabolism in animals and humans and emerging evidence for important biological functions of vitamin E metabolites made it necessary to enhance the existing analytical procedures for vitamin E [40]. All vitamin E forms undergo enzymatic modification (sulfation and glucuronidation) during their metabolic degradation, complicating their detection in biological samples. Thus, only a few research groups were able to establish valid methods for the determination of vitamin E metabolites in human matrices (reviewed in [40]), and so far only a few detected the LCMs in human blood [10, 11, 24, 41].

The different physicochemical properties of the vitamin E metabolites are major criteria for the development of appropriate analytic strategies. Short-chain metabolites (SCMs) and LCMs were found as sulfates, glucuronides, and glucosides or as unconjugated carboxychromanols in different matrices [40]. Therefore, the quantitative extraction of these metabolites from biological samples requires enzymatic deconjugation using sulfatase and β-glucuronidase or chemical hydrolysis.

Table 6.1 Subset of analyzable metabolites in biological samples with their corresponding preparation and detection methods

Deconjugation	Extraction	Chromatography	Sample	Metabolite	Ref.
–	LL	GC-MS	Cell culture supernatant (HepG2)	γ-7′,9′,11′,13′-COOH γ-13′-OH	[43]
S	LL	HPLC-FLD LC-MS (ESI(−))	A549 cells	γ-9′,11′13′-COOH γ-13′-OH γ-9′S,11′S,13′S-COOH δ-9′,11′,13′-COOH δ-9′S,11′S,13′S-COOH	[44]
			Rat plasma (200 µl)	γ-9′S,11′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
			Rat liver (homogenate of the entire organ)	γ-9′S,13′S-COOH γ-13′-COOH, γ-13′-OH α-TOH, γ-TOH	
–	–	HPLC-UV-Vis	Cell culture supernatant (HepG2)	α-5′,13′-COOH α-13′-OH	[48]
S + G	LL	HPLC-ECD LC-MS (ESI(−))	Human serum (20 µl)	ICM, SCM, TOH	[49]
			Human urine (20 µl)	ICM, SCM	
			Human feces (10 mg)	α-3′,5′,13′-COOH γ-3′,11′-COOH δ-3′,11′-COOH α-TOH, γ-TOH, δ-TOH α-T3, γ-T3	
S + G	LL	Q-TOF-LC-MS (ESI(+))	Human serum (500 µl)	α-13′-COOH	[11]
–	LL	HPLC-ECD GC-MS	Human serum (1 ml)	α-13′-OH	[10]
n.i.	LL	LC-MS/MS (APCI)	Human serum (1 ml)	α-13′-COOH α-13′-OH	
S + G	LL	LC-MS/MS (ESI(+))	Human plasma or serum (500 µl)	α-3′,13′-COOH γ-3′-COOH α-13′-OH	[24]
			Human plasma or serum (100 µl)	α-TOH, γ-TOH	

Abbreviations used are the following: *n.i.* no information, *LL* liquid-liquid, *GC* gas chromatography, *HPLC* high-pressure liquid chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *FLD* fluorescence detector, *S* sulfatase, *G* β-glucuronidase, *ESI* electrospray ionization, *APCI* atmospheric pressure chemical ionization, *SCM* short-chain metabolite, *ICM* intermediate-chain metabolite, *T3* tocotrienol, *TOH* tocopherol, *OH* hydroxychromanol, *COOH* carboxychromanol

The SCMs have been measured in urine, plasma, feces, cell extracts, and other biological fluids (e.g., bile), with analytical strategies adopted to their chemical characteristics, including water solubility and chemical conjugation. In contrast, the LCMs exhibit a more lipophilic nature and thus occur in feces, cells, tissues, and blood but not in urine. Hence, analytical strategies for the LCMs focus on their lipophilic properties and their occurrence as sulfated derivatives (especially the carboxychromanols) [40]. A brief overview of a subset of analyzable metabolites and the corresponding methods for preparation and detection is provided in Table 6.1.

Specifications of Long-Chain Metabolites

The vitamin E metabolites with a side chain length between 13 and 9 carbon units are summarized as LCMs, with 13′-OH and 13′-COOH being the first metabolites formed from their metabolic precursors, the TOHs or tocotrienols (T3s) [42]. The first analytical approach for the detection of γ-13′-OH,

γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH has been published in 2002 by Sontag and Parker, who used a GC-coupled MS-based approach for the determination of these metabolites in the culture supernatant of HepG2 liver cells that have been incubated with *RRR*-γ-TOH [43]. This finding was confirmed by Jiang and coworkers who detected γ- and δ-LCMs in the cell culture supernatant of human lung epithelial A549 cells. Especially the carboxychromanols were detected in their sulfated form, indicating that acid metabolites are preferred for this type of chemical modification [44]. The sulfate modification can be removed by enzymatic deconjugation, leading to facilitated detection and improved analytical recovery of the acid metabolites [41]. Hence, Jiang and coworkers detected for the first time the γ-LCMs in complex matrices, such as rat liver and plasma [44]. At about the same time when Sontag and Parker published their first analytical approach for the detection of the γ-LCMs, Azzi and coworkers discovered gene regulatory actions of α-TOH, a finding that was also confirmed for the corresponding LCMs [45, 46]. Further, recent insights into the metabolism of vitamin E showed that *RRR*-α-TOH is the preferred form for hepatic uptake, rendering α-TOH as the most relevant vitamin E form with biological activity in humans [42, 47]. Based on these findings, the focus of analytical interest switched from the γ- to the α-TOH LCMs. In 2010, Birringer and coworkers detected for the first time α-13′-OH and α-13′-COOH in the culture supernatant of HepG2 liver cells enriched with *RRR*-α-TOH [48].

In the same year, Mustacich and coworkers used a LC-MS technique to analyze α-13′-OH in rat liver microsomes, presenting the first determination of an α-LCM in a complex matrix [2]. Further, the first determination of the α-LCMs in humans was done by the group of Zhao et al., who detected α-13′-COOH in human feces [49]. In 2014, 12 years after Azzi and coworkers published their hypothesis outlining a gene regulatory role for α-TOH, Wallert et al. found α-13′-COOH in human serum, indicating a systemic relevance in humans. In this study, 500 µl serum of a healthy, middle-aged (39 years), nonsmoking male, who received a balanced diet with no additional supplementation of vitamin E, was used for the detection of α-13′-COOH via quadrupole time-of-flight (Q-TOF) LC-MS [11]. This study provided first evidence that the α-LCMs are transferred into blood circulation after α-TOH has been metabolized in the liver. Further, Wallert and coworkers showed in a cell model that α-13′-OH and α-13′-COOH are more potent regulators of gene expression than their metabolic precursor [11]. Taken together the results of Wallert et al. indicate that the LCMs are a more active form than their precursor molecules that might promote regulatory effects in peripheral tissues of the human body. Only 1 year later, α-13′-OH was also found in human serum using a GC-MS approach [10]. Based on these two analytical approaches, current analytical research is focused on the development of analytical strategies enabling the simultaneous determination of all LCMs and their respective metabolic precursors. The first attempt was made in 2016, when Torquato et al. used LC-MS/MS combined with atmospheric pressure chemical ionization (APCI) (−) for the detection of α-13′-OH and α-13′-COOH in the same analytical session [41]. This analytical approach has been confirmed by Giusepponi and coworkers by using LC-MS/MS with a different type of ionization (electrospray ionization (ESI) (+)) [24]. In 2012, Bardowell and coworkers were able to determine 12′-OH and 11′-OH in the feces of mice fed a γ-TOH-enriched diet [50]. The detection of these metabolites in feces provided evidence for ω-1 and ω-2 hydroxylation activity and that 12′-OH cannot undergo oxidation followed by side chain truncation. Therefore, this metabolite is excreted via bile and can be found in the feces of mice and humans [50].

Sample Preparation

In the early days of vitamin E research, solid extraction of lipid fractions from biological matrices was the only challenge in a preparation procedure. With the growing knowledge about chemical properties, metabolic pathways, and tissue distribution of vitamin E metabolites, the challenges got more difficult and complex. Today, issues such as conjugation, oxidation, chemical differences between the single compounds, and even their appearance in various matrices must be considered. While the LCMs can be detected in feces, cells, tissues, and blood, the SMCs are mostly found in biological

fluids, such as bile, urine, and plasma, but also in feces, isolated cells, and solid tissues [40]. To increase the recovery of vitamin E metabolites, cell and solid tissue samples must be homogenized, and complex lipids need to be hydrolyzed before lipid extraction. During these processes, antioxidants like butylated hydroxytoluene, ascorbic acid, and pyrogallol can be added to avoid autoxidation of the metabolites [11, 51].

The use of analytical standards is essential to assess metabolite recovery during workup and analysis. Therefore, authentic compounds or stable isotope-labeled synthetic analogues need to be added at the beginning of the sample preparation. In the case of the 13′-OH and 13′-COOH LCMs, no analytical standards are commercially available, and the molecules must be synthesized or semi-synthesized from natural compounds [34, 48, 52]. Therefore, Wallert and coworkers used garcinoic acid, a natural compound occurring in the nuts of the African plant *Garcinia kola* (reviewed in [53]) for the semi-synthesis of α-13′-COOH and α-13′-OH as well as δ-13′-COOH and δ-13′-OH [11].

Another important step of the preparation procedure is the enzymatic deconjugation of the vitamin E metabolites. The LCMs as well as the SCMs mostly appear as sulfated or glucuronidated conjugates in biological samples [11, 51]. These conjugates are a result of the chemical modification during the hepatic metabolism and can lower the recovery of their corresponding metabolites. Freiser and coworkers reported that especially the acid forms of the LCMs are conjugated, with a predominance of the sulfate conjugates [54]. This mismatch between sulfation and glucuronidation has been confirmed in various studies, indicating that enzymatic sulfation could be the predominant phase II reaction in vitamin E metabolism [55–57]. The enzymatic hydrolysis of conjugated metabolites with β-glucuronidase and sulfatase appeared as a reliable method for the workup of biological fluids or tissues in different publications [11, 51]. Wallert and coworkers incubated 500 µl human serum for 30 min (at 34 °C) with a combination of 1500 IU β-glucuronidase and 26 IU/ml sulfatase for enzymatic deconjugation. This procedure led to a higher recovery of the unconjugated acid LCMs, enabling the first determination of α-13′-COOH in human serum [11]. In addition, the application of methanolic HCl for the deconjugation of CEHCs in urine samples appeared to be more efficient than enzymatic hydrolysis [56]. Beneath the strategy of enzymatic deconjugation, Pope and coworkers tried to analyze the conjugates directly using MS (ESI) to avoid artificial production of Simon metabolites by deconjugation steps. This method showed promising results for CEHC but has to be improved further for the general determination of vitamin E metabolites [58].

In most analytical studies on vitamin E, liquid-liquid extraction was used for the purification of the metabolites and their metabolic precursors from different matrices [10, 11, 24, 44, 49]. Wallert and coworkers performed liquid-liquid extraction with a mixture of hexane and dichloromethane (ratio 5:2) containing 1% butylated hydroxytoluene. The serum samples were mixed with solvent for 1 min at room temperature and were then centrifuged (2000 × g, 15 min, 10 °C) to achieve the separation of organic and inorganic layers. The upper organic phase was collected in glass tubes, dried under N_2, and resuspended in 50 µl methanol [11]. Solid-phase extraction (SPE) is another way to extract vitamin E metabolites from biological matrices. Yang et al. isolated γ-13′-OH, γ-13′-COOH, γ-11′-COOH, and γ-9′-COOH from cell culture medium of A549 cells with a C_{18}-SPE cartridge, using acetic acid for metabolite elution [59]. Next, Wallert and coworkers are currently working on a SPE-based method for the extraction of the LCMs from human blood (unpublished data). Here, only 100 µl plasma will be used for metabolite extraction, providing a significant advantage compared to the liquid-liquid extraction-based alternatives, which require 500 µl plasma.

Detection of Vitamin E Long-Chain Metabolites

LC-/HPLC Analysis

HPLC-based analysis of vitamin E metabolites is performed with either normal phase or reverse phase columns coupled to electrochemical (ECD), fluorescence (FLD), UV-Vis, or evaporative light scattering detectors, with ECD being the most sensitive for vitamin E determination. Therefore,

HPLC-ECD has been used by Zhao and coworkers to detect TOH metabolites in human feces [49]. Fluorescence detection has lower sensitivity than ECD, and the response for some of the physiological metabolites is too low for applications involving human samples. However, FLDs have been also used for the determination of TOHs, T3s, and their corresponding metabolites in cell culture supernatants [44], rat plasma and liver [44, 54], and fetal bovine serum [57]. LC-MS/MS is the most widely used technique for the determination of the LCMs, providing an accurate quantitative analysis of these compounds in various biological matrices [24, 41, 57, 60]. Hence, LC-MS/MS enabled the first simultaneous detection of α-13′-OH and α-13′-COOH in human serum [1]. Further, Jiang et al. showed that negative polarity (ESI(−)) LC-MS/MS can be also used to quantify conjugated and unconjugated vitamin E metabolites in rodent blood [57].

GC Analysis

GC-based methods for the analysis of vitamin E are either coupled to flame ionization detectors (FID) or MS detectors. In contrast to HPLC-based methods, GC-based analysis of vitamin E metabolites requires an additional derivatization step for TOH and carboxychromanols [43, 51]. The purified extracts are heated and silylated with N-methyl-N-trimethyl-silyltrifluoroacetamide (MSTFA) or N,O-(bis-trimethylsilyl) trifluoroacetamide (BSTFA) to accomplish derivatization [61]. This additional procedure is required for the detection of TOH and its metabolites by GC-based separation and detection. Traditionally, FID is the most often used detector in GC, due to its high response to organic molecules, but nowadays this detection technique is more and more replaced by MS, allowing a more sensitive detection of vitamin E metabolites. Therefore, GC-MS-based methods have been used for metabolite detection in cell culture supernatants [43, 44], the simultaneous detection of α-TOH and its oxidation product α-tocopherolquinone in human blood [61], and the first determination of α-13′-OH in human serum [10]. Further, GC-MS procedures were also used to investigate Simon's metabolites and α-CEHC in plasma and urine of animals and humans [62, 63], as well as for the analysis of α- and γ-TOH with their corresponding metabolites in human plasma [51].

Features of Long-Chain Metabolite Analysis in Human Blood

In recent years, the LCMs have emerged as a new class of signaling molecules with possible relevance for the regulation of physiological functions. This change of direction in vitamin E research resulted in the development of new analytical methods to assess the LCMs in human matrices. Unfortunately, the determination of these compounds, especially in blood, appeared to be very difficult. The first detection of the α-LCMs in human serum was in 2014 by Wallert and coworkers. This group detected α-13′-COOH, but not its metabolic precursor α-13′-OH, in the serum of a healthy volunteer, receiving 1000 IU of *RRR*-α-TOH/day over 1 week. Before the measurement, the LCMs undergo enzymatic deconjugation with a mixture of sulfatase and β-glucuronidase and were extracted with hexane and dichloromethane. Based on the results of Wallert et al., the LCMs seem to appear in low nanomolar concentrations in human serum, indicating that detection sensitivity could be a major problem for metabolite analysis in future studies [11]. Only 1 year later, Ciffolilli et al. determined α-13′-OH in the same serum sample with a GC-MS-based method. Again, the applied method could not be used to detect also the second α-LCM (α-13′-COOH) [10]. To overcome these drawbacks, Torquato and coworkers tried to optimize the proposed methods by using LC-MS/MS. First, APCI and ESI sources were compared in positive and negative acquisition mode for the simultaneous determination of the TOHs and the LCMs with APCI (−) providing the best signal intensity for the α-LCMs [41]. As a result of the optimized protocol, Torquato and coworkers were able to detect α-13′-OH and α-13′-COOH simultaneously in one serum sample [41]. Only 1 year later, Giusepponi and colleagues obtained the same results using an ESI (+) source [24]. Interestingly, both groups were for the first time able to separate α-13′-OH and α-13′-COOH from several unknown compounds with identical

masses. An accurate mass investigation performed by Giusepponi et al. identified these unknown compounds as possible structural isomers of α-13′-OH and α-13′-COOH. If this holds true, the blood concentrations of α-13′-OH and α-13′-COOH would be detected as a bulk parameter, comprised of up to three different isomers [24, 41].

Regulatory Actions of Vitamin E Long-Chain Metabolites

Anti-inflammatory Actions

For studies on the anti-inflammatory actions of the LCMs, (i) cells were treated with the respective LCM in conjunction with a pro-inflammatory stimulus, or (ii) isolated enzymes were used to study the influence of the LCMs on their activity. Several LCMs (α-, γ-, δ-13′-COOH; δ-9′-COOH; α-13′-OH) affected the inflammatory response, i.e., expression (mRNA or protein) or the activity of various pro-inflammatory enzymes, including cyclooxygenase 2 (COX2) [10, 34, 64, 65], inducible nitric oxide synthase (iNOS) [10, 65–67], or 5-lipoxygenase (5-LO) [64, 68], as well as inflammatory mediators such as chemokines and cytokines. In general, the 13′-COOH metabolites are more potent than the shorter LCMs, and the conjugation of the LCMs with sulfate abrogates their anti-inflammatory actions [34, 67].

The first study on the anti-inflammatory actions of the LCMs was carried out in 2008 by Jiang and coworkers in human adenocarcinomic alveolar basal epithelial cells. This cell line is capable to metabolize vitamin E and showed an inhibition of the arachidonic acid-stimulated COX activity after treatment with TOH [34]. The inhibitory effect was less effective after pre-treatment with sesamin, a known suppressor of the metabolism of vitamin E, indicating an involvement of the LCMs as regulatory substances. In addition, the LCMs were extracted from the cell culture supernatant of the A549 cells to confirm their inhibitory capacity on COX activity (IC_{50}: δ-13′-COOH: 4 μM; δ-9′-COOH: 6 μM). The same experiments were performed with the sulfated LCM conjugates, which did not exert anti-inflammatory effects, indicating that only unconjugated LCMs can act as anti-inflammatory compounds [34]. Anti-inflammatory actions on lipopolysaccharide (LPS)-stimulated COX2 mRNA and protein expression as well as release of COX-derived prostaglandins PGE_2 for α-13′-OH [10] and PGE_2, PGD_2, and PGF_{2a} for α-13′-COOH [65] were also shown in murine RAW264.7 macrophage-like cells.

In addition, the α- and δ-LCMs (α- and δ-13′-OH, α- and δ-13′-COOH) mediated the inhibition of iNOS mRNA and protein expression, as well as release of NO in response to LPS in RAW264.7 macrophages [10, 65–67]. Interestingly, the observed inhibitory effects depended on the structure of the LCMs, with the 13′-COOH metabolites being more effective than the 13′-OH metabolites, while the substitution of the chroman ring (α- vs. δ-LCMs) had no detectable influence.

The inhibitory effects of the LCMs on 5-LO activity have been shown in (i) human promyelocytic HL60 leukemia cells, where the LCMs blocked the ionophore-induced release of leukotriene B_4, as well as (ii) on the isolated 5-LO, where δ-13′-COOH was more effective than zileuton, a synthetic antagonist for 5-LO [68]. The inhibition of 5-LO activity by δ-13′-COOH was also reported by Jang et al. [64].

Cellular Lipid Homeostasis

Until now, only a few aspects of lipid homeostasis have been studied regarding their modulation by the LCMs. Hence, merely these aspects can be discussed in the following. These include the regulation of cluster of differentiation 36 (CD36), uptake of oxidized LDL, phagocytosis, and the

intracellular storage of lipids. Taken together, these mechanisms represent key processes in macrophage foam cell formation, a significant hallmark of the pathogenesis of atherosclerosis [69]. The human monocytic THP-1 cell line, which can be differentiated to macrophage-like cells, was used to study the effects of the LCMs on foam cell formation by Wallert et al. in 2014 [11]. Under basal conditions (i.e., without the stimulation with oxidized LDL), the LCMs α-13′-OH and α-13′-COOH induced the expression of CD36 mRNA as well as CD36 protein. Interestingly, this result contrasts with the effects of the precursor α-TOH, which downregulated the expression of CD36 at a concentration of 100 μM in the THP-1 macrophage model. Thus, the metabolites likely function in a different mode than their natural precursors. In addition, the LCMs appear to be several times more effective than α-TOH, as they exert their effect on CD36 expression in concentrations as low as 5 and 10 μM for α-13′-OH and α-13′-COOH, respectively. To confirm these findings in a more physiological model, the LCMs were also applied to peripheral blood mononuclear cell (PBMC)-derived primary macrophages in this study. Here, the effects of both LCMs on CD36 protein expression were confirmed [11].

The scavenger receptor CD36 is a receptor binding oxidized LDL in macrophages and mediates the uptake of this modified lipoprotein [70]. In a feed-forward mechanism, oxidized LDL induces the expression of CD36, leading to an increased uptake of oxidized LDL [71]. This mechanism promotes foam cell formation; thus, Wallert et al. examined whether the LCMs interrupt or support oxidized LDL-induced CD36 expression. As expected, the incubation with oxidized LDL induced the expression of CD36 in human THP-1 macrophages [11]. The TOH precursor diminished the induction by oxidized LDL, resembling the findings under basal conditions. Likewise, the effect of the LCMs resembled the initial findings. The preincubation with the LCMs augmented the induction of CD36 protein expression by oxidized LDL significantly. In contrast, naïve LDL did not induce CD36 expression in THP-1 macrophages. In combination with naïve LDL, α-TOH downregulated CD36 protein expression, while the LCMs induced the expression. Given the augmented expression of CD36 by the LCMs, increased oxidized LDL uptake by LCM-treated macrophages can be expected. Interestingly, incubation of THP-1 macrophages with the LCMs for 24 h before challenging the cells with oxidized LDL leads to a decrease of about 20% in the uptake of oxidized LDL compared to untreated control cells. Again, PBMC-derived macrophages were treated in a similar fashion, and the findings were confirmed. The LCM α-13′-OH decreased the uptake by 24% and α-13′-COOH by 20%, respectively. In foam cell formation, the consequence of an increased uptake of oxidized LDL is an increase in intracellular lipid content. Thus, the THP-1 macrophage model reacted with an increase of intracellular neutral lipids in response to the incubation with oxidized LDL. Concomitant with the decreased uptake of oxidized LDL in response to the LCM preincubation, the neutral lipid content of THP-1 macrophages was not increased in cells treated with the LCMs and oxidized LDL in combination [11].

However, the induced expression of CD36 is contradictory to the observed inhibitory effects of the LCMs on the uptake of oxidized LDL. Consequently, the LCMs likely act through a distinct mechanism. Results by Wallert et al. suggest that phagocytosis, as a major uptake pathway for oxidized LDL [72], is also affected by the LCMs. Experiments with fluorescence-labeled microbeads revealed that the LCMs significantly decreased the phagocytic activity of THP-1 macrophages. Here, α-13′-COOH seems to be more potent than α-13′-OH, with 41% inhibition vs. 16% inhibition, respectively [11]. This finding is not perfectly in line with the equal inhibitory effect of the two LCMs on the uptake of oxidized LDL. However, the inhibition of phagocytosis by the LCMs provides a good explanation for the discrepancy between CD36 regulation and uptake of oxidized LDL.

Taken together, the LCMs modulate macrophage lipid metabolism on the level of lipid uptake and storage. Different pathways implicated in foam cell formation, a hallmark of atherosclerosis, are affected by the LCMs. In total, the treatment of macrophages with the LCMs leads to a reduced uptake of oxidized LDL and concomitantly reduced lipid accumulation, a desirable effect in terms of the prevention of atherosclerosis. However, the underlying molecular mechanisms are not fully understood. Thus, further studies on the modes of action of the LCMs are needed.

Cancerogenesis and Chemoprevention

Antiproliferative Effects of Tocopherol Long-Chain Metabolites

Abnormal proliferation is a characteristic of cancer cells and represents a crucial element of cancer development and progression. Thus, cancer therapy is based in part on drugs that kill cells with high rates of proliferation and regeneration. However, such substances cause severe side effects as they also affect rapidly proliferating healthy tissues like the skin, hair, or parts of the gastrointestinal tract [73]. Hence, natural compounds with antiproliferative activities are regarded as beneficial in the prevention and treatment of cancers, as they generally exert less side effects. Several natural compounds have been identified that inhibit pathways contributing to cell proliferation. Among them are the promising constituent of *Curcuma longa*, namely, curcumin, which has been shown to affect Wnt, NF-κB, and mTOR signaling inter alia and resveratrol, a constituent of grapes, with blocking activity on mitogen-activated protein kinases and tyrosine kinases inter alia [73]. The chemopreventive properties of curcumin [74] and resveratrol [75] have been found in several studies. Cancer-preventing properties have also been reported for TOHs and T3s. This effect can be attributed at least in part to the LCMs as outlined below.

First studies on the TOH metabolites in this context were carried out with a focus on the SCMs. Here, the SCMs exerted comparable effects to their precursors with respect to the inhibition of cell proliferation. Interestingly, the γ-forms appeared to be more potent in inhibiting cell proliferation than the α-forms, a finding that was earlier reported for the T3s [76]. Accordingly, it was found that γ-TOH as well as γ-CEHC reduced the proliferation of human PC3 prostate cancer cells in a concentration of 1 μM by about 30–40%. Almost maximal inhibition of cell proliferation, i.e., 70–80%, was obtained with 10 μM. However, α-CEHC and α-TOH inhibited cell growth by 40–45% in concentrations of 50 μM [77]. Interestingly, the antiproliferative effects seem to be cell type-dependent, as PC3 cells showed higher inhibition compared to human HTB-82 rhabdomyosarcoma cells and human endothelial vascular cells (HEVC) [77]. Given that the precursors and the SCMs exert antiproliferative effects, Birringer et al. were interested in the effects of the LCMs on cell proliferation. Therefore, α-13′-COOH and δ-13′-COOH as well as α-13′-OH and δ-13′-OH and their respective precursors were applied to HepG2 liver cells [48]. While the LCMs with carboxy function potently led to cell growth arrest, the hydroxy metabolites failed to exert antiproliferative effects. Again, the α-forms were less potent than their δ-counterparts. Neither the hydroxy metabolites nor the TOHs inhibited cell growth in the concentrations tested. Thus, the authors concluded that the carboxylation of the TOH side chain is essential for the antiproliferative effects of the LCMs [48]. However, in PC3 cells, not only α- and γ-CEHC impeded cell proliferation but also δ-13′-COOH and α-13′-OH. All compounds inhibited cell proliferation by about 60% at concentrations of 10 μM [52]. Thus, the effect of the hydroxy metabolites seems to be cell type-dependent. This might be explained by differences in the cellular metabolism of the TOHs and the TOH metabolites. Different responsiveness of cell types to vitamin E metabolites was also reported in colon cells [64]. The δ-13′-COOH metabolite reduced the proliferation of human HCT-116 colon carcinoma and human HT-29 colorectal adenocarcinoma cells with IC_{50} values of 8.9 μM and 8.6 μM, respectively, whereas the T3 metabolite δ-T3-13′-COOH (i.e., δ-garcinoic acid) was less potent with IC_{50} values of 16 μM and 17 μM. Interestingly, normal colon epithelial cells were less affected by the metabolites. While 10 μM of δ-13′-COOH suppressed cell viability of HCT-116 and HT-29 cells by around 60%, normal human colon epithelial cells showed reduction of merely 10–20%. In line with this, 20 μM of δ-T3-13′-COOH (δ-garcinoic acid) reduced the viability of the cancer cells by 70–80%, but the viability of normal colon cells was affected only by 10–20% [64].

Taken together, the precursor molecules, i.e., the TOHs and T3s, apparently exert antiproliferative effects depending on the methylation pattern of the chroman ring. The γ-forms of T3 and TOH have antiproliferative properties [76, 77], while the α- and δ-forms have not [48, 64]. It should be noticed that Jang et al. found no effects of γ-T3 in their setting [64]. However, the metabolic conversion leads to

LCMs and SCMs with antiproliferative properties, independent of the methylation pattern of the chroman ring. While the action of the hydroxy LCMs is controversial [48, 52] and likely depends on the cell type, the carboxy LCMs reliably affect the proliferation of different cancer cell lines [48, 52, 64]. Thus, a key determinant of the antiproliferative properties is likely the carboxy function, a notion that is further supported by the reported actions of the SCMs carrying a carboxy group (α- and γ-CEHC) [52, 77]. A promising finding with respect to anticancer properties of the LCMs is the resistance of normal colon cells to the LCMs, while the proliferation of colon cancer cells is strongly reduced [64]. If this effect is reproducible, vitamin E and its metabolites might be useful in cancer prevention and treatment.

Pro-apoptotic Effects of the Tocopherol Long-Chain Metabolites

Apoptosis is a coordinated cellular process, ultimately leading to programmed cell death. The balance of cell division and cell death is crucial for the homeostasis of organisms. Inappropriate rates of apoptosis are implicated in several pathological conditions, such as neurodegenerative diseases, autoimmune disorders, and cancers [78]. The rate of apoptosis is usually lower in cancer cells, leading to malignant cells, tumor metastasis, and resistance to anticancer drugs. Thus, apoptosis is part of the problem as well as a possible solution. Several therapeutic strategies based on the targeting of apoptosis pathways have been developed [78].

In addition to the antiproliferative effects, Birringer et al. also analyzed the apoptotic effects of the LCMs in HepG2 liver cells [48]. Flow cytometric analyses using annexin V staining revealed a significant induction of apoptosis, when HepG2 cells were treated with 20 μM α-13′-COOH, δ-13′-COOH, or δ-13′-OH. In line with this, α-13′-COOH and δ-13′-COOH strongly induced the cleavage of caspases 3, 7, and 9. The hydroxy LCM δ-13′-OH leads to an activation of the same caspases but less effectively. In contrast, α- and δ-TOH as well as α-13′-OH were not able to induce caspase cleavage. Accordingly, poly-ADP ribose polymerase (PARP)-1 cleavage as a downstream effect of caspase activation followed a similar pattern. The α- and δ-carboxy metabolites showed strong induction, while merely a slight effect for δ-13′-OH and no effect for α-13′-OH and the TOHs were observed. Further, mitochondrial apoptosis, a process accompanied by the increased production of reactive oxygen species (ROS), was examined. On that account, ROS production in the HepG2 cells in response to TOHs and their metabolites was analyzed. Here, in contrast to the precursors and the hydroxy metabolites, the carboxy metabolites significantly induced ROS formation. Not only intracellular but also intramitochondrial ROS levels were induced by the carboxychromanols. Again, the other substances tested did not induce ROS production. With these findings, evidence was provided for mitochondrial-derived apoptosis. Further, alterations in the mitochondrial membrane potential in TOH- and LCM-treated cells were found. Significant reductions in the mitochondrial membrane potential were observed for α-13′-COOH, δ-13′-COOH, and δ-13′-OH in concentrations of 20 μM. Here, α-13′-COOH was again more effective (60% reduction) than the δ-LCMs (20% reduction for both the hydroxy and the carboxy metabolites) [48].

Taken together, the carboxy LCMs reliably induce apoptosis in HepG2 cells, and evidence was provided that a pathway leading to mitochondrial apoptosis is involved in this effect. Interestingly, the authors have shown that δ-13′-OH is efficiently metabolized to δ-13′-COOH by HepG2 cells, while the conversion of α-13′-COOH to α-13′-OH is less effective [48]. This finding provides a nice explanation for the discrepancy in the effects of α-13′-OH and δ-13′-OH. The δ-metabolite leads to apoptosis through a rapid conversion to the pro-apoptotic carboxy metabolite, while the α-metabolite is slowly converted and thus unable to induce apoptosis. In conclusion, as shown for the antiproliferative actions of the LCMs, the carboxy function of the metabolite seems to be crucial for the observed pro-apoptotic effect.

The apoptotic actions of the long-chain vitamin E derivatives with carboxy function were confirmed in a study on colon cancer cells [64]. Here, δ-13′-COOH and δ-T3-13′-COOH (δ-garcinoic acid) induced early and late apoptosis. In line with the findings of Birringer et al., induction of

caspase-9 activation and PARP cleavage by the carboxy metabolites was found. Further, the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-II was increased by the treatment with the carboxy LCMs. Interestingly, the TOH metabolite was more effective than the T3 metabolite in the induction of apoptosis and autophagy. Based on previous findings on the metabolic precursors, Jang et al. examined whether an alteration in sphingolipid metabolism by the LCMs is causing the induction of apoptosis. It was found that δ-13′-COOH increased dose-dependently the total content of ceramides, dihydroceramides, and dihydrosphingosines. In contrast, the content of all sphingomyelins was decreased. Similar effects were observed for the T3 metabolite. Thus, both carboxy LCMs modulate sphingolipid metabolism when apoptosis and autophagy are induced. An inhibition of sphingosine biosynthesis by myriocin treatment partly inhibited the induction of LC3-II expression but not the induction of PARP cleavage by the metabolites. Hence, elevated levels of dihydroceramides and dihydrosphingosines likely contribute to LCM-induced autophagy [64].

The LCMs have been shown to induce apoptosis in different cell types. Interestingly, evidence for two different modes of action has been provided by the studies on the LCMs so far. Birringer et al. have shown the induction of mitochondrial apoptosis by the LCMs in HepG2 cells, while Jang et al. have reported that an altered sphingolipid metabolism contributes to LCM-induced apoptosis in colon carcinoma cells. Treatment strategies targeting apoptosis aim at different signaling pathways, including B-cell lymphoma proteins, p53, or caspases. Effects of the LCMs on caspases have been shown in both studies on LCM-induced apoptosis. However, based on these findings, the applicability of the LCMs for chemoprevention or inhibition of cancerogenesis can hardly be assessed. Further studies are required to confirm desired properties like specificity for malignant cancer cells or to unravel distinct apoptosis signaling pathways [78].

Interaction with Pharmaceuticals

The cellular uptake of molecules is tightly regulated by several mechanisms, one of which is the excretion of, for example, pharmaceuticals from the cells via exporter proteins, such as the multidrug resistance protein P-gp. P-gp is a well-known representative of these exporters [79]. A specific inhibition in tumor cells is helpful, when antitumor therapies are applied, since the activation of these exporters may lead to a reduced cellular net uptake and efficiency of the pharmaceuticals.

Podszun et al. studied the effects of vitamin E (α-TOH, α-T3, γ-TOH, and γ-T3) and their metabolites (α-13′-COOH, α-CEHC, γ-CEHC) as well as plastochromanol-8 on the expression of P-gp in LS 180 Dukes' type B colorectal adenocarcinoma cells and found an induction of the expression and activity for α-13′-COOH and γ-T3 [80]. Furthermore, pregnane X receptor activity was induced by α-T3, α-13′-COOH, and γ-T3, as assayed by a reporter gene assay. The authors summarized that an increased uptake of vitamin E via supplements could lead to interactions with pharmaceuticals due to an increased activity of the P-gp exporter.

Structure-Specific Effects

A structure-function relationship study of the LCMs revealed a highly specific regulation of target genes by the LCMs (α-13′-OH, α-13′-COOH, δ-13′-OH, and δ-13′-COOH). Neither the precursors (α- and δ-TOH) nor their substructures (pristanic acid and α-CEHC) were able to cause the same effects on the expression of scavenger receptor CD36 or inducible nitric oxide synthase (iNos) as the 13′-hydroxy or 13′-carboxy LCMs. Furthermore, the regulation was almost independent of the substitution pattern of the chromanol ring system (α- vs. δ-LCMs) but dependent on the modification of the side chain (TOH vs. 13′-OH and 13′-COOH, respectively), with the 13′-COOH being most potent. Hence, this specific regulation might suggest the existence of receptor-specific pathways for the LCMs [67].

Conclusions and Outlook

With the demonstration of the occurrence of the LCMs in human serum, Wallert and coworkers provided evidence for their possible role as systemic signaling molecules [11]. This concept was supported by several studies, characterizing the involvement of the LCMs in the regulation of inflammatory processes, lipid metabolism, cancerogenesis, and chemoprevention as well as xenobiotic metabolism. Interestingly, the LCMs act more potent and in part even contrary to their precursors. Thus, some of the controversial effects found for vitamin E might be explained by the actions of the LCMs. Nevertheless, large parts of their mode of action are still unrevealed and need further characterization. Although the analysis of the LCMs made great progress over the last several years, especially the distribution of the LCMs in extrahepatic tissues beside human serum needs further investigation. Taken together, the LCMs could be regarded as the active forms of vitamin E, as it has already been shown for the metabolites of vitamin A and D (reviewed in [81]). If this concept of a general mechanism for metabolic activation of fat-soluble vitamins holds true, the LCMs of vitamin E could comprise a new class of signaling molecules in the human body. This concept sheds new light to the field of vitamin E research and may help for better understanding the complex mode of action of vitamin E as well as its function as a vitamer. A brief overview about the current knowledge on LCMs in the human body and issues for future investigations is provided in Fig. 6.1.

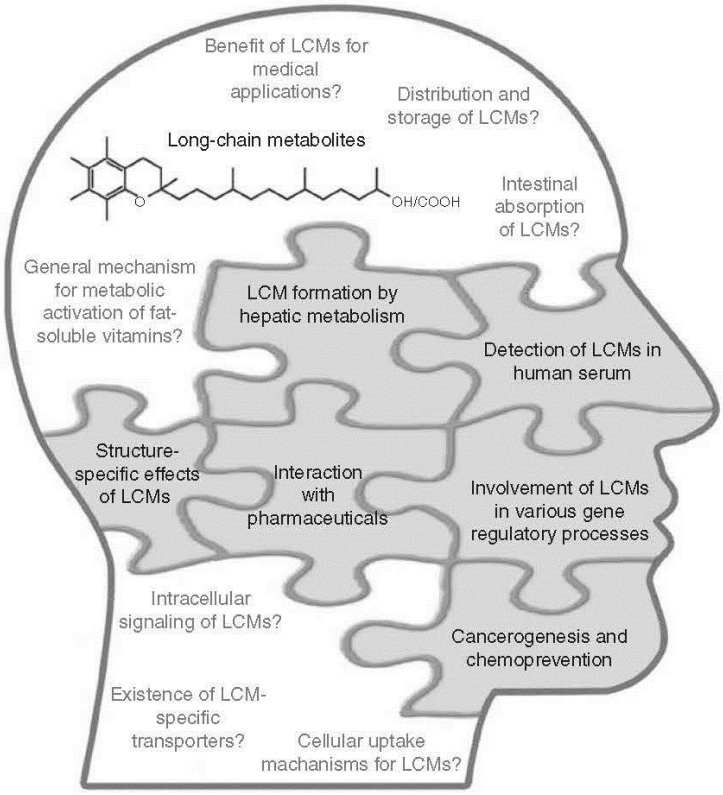

Fig. 6.1 Completing the puzzle of the activities of the LCMs in the human body

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7.10 Manuscript X

frontiers in Pharmacology

REVIEW
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Diversity of Chromanol and Chromenol Structures and Functions: An Emerging Class of Anti-Inflammatory and Anti-Carcinogenic Agents

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Natural chromanols and chromenols comprise a family of molecules with enormous structural diversity and biological activities of pharmacological interest. A recently published systematic review described more than 230 structures that are derived from a chromanol ortpd chromenol core. For many of these compounds structure-activity relationships have been described with mostly anti-inflammatory as well as anti-carcinogenic activities. To extend the knowledge on the biological activity and the therapeutic potential of these promising class of natural compounds, we here present a report on selected chromanols and chromenols based on the availability of data on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis. The chromanol and chromenol derivatives seem to bind or to interfere with several molecular targets and pathways, including 5-lipoxygenase, nuclear receptors, and the nuclear-factor "kappa-light-chain-enhancer" of activated B-cells (NFκB) pathway. Interestingly, available data suggest that the chromanols and chromenols are promiscuitively acting molecules that inhibit enzyme activities, bind to cellular receptors, and modulate mitochondrial function as well as gene expression. It is also noteworthy that the molecular modes of actions by which the chromanols and chromenols exert their effects strongly depend on the concentrations of the compounds. Thereby, low- and high-affinity molecular targets can be classified. This review summarizes the available knowledge on the biological activity of selected chromanols and chromenols which may represent interesting lead structures for the development of therapeutic anti-inflammatory and chemopreventive approaches.

Keywords: chromanols, chromenols, inflammation, cancer, molecular targets

INTRODUCTION

Chromanols and chromenols are collective terms for about 230 structures derived from photosynthetic organisms like plants, algae, cyanobacteria, fungi, corals, sponges, and tunicates (Birringer et al., 2018). Both compound classes are formed by a cyclization of substituted 1,4-benzoquinones. While 6-hydroxy-chromanols are derived from a 2-methyl-3,4-dihydro-2*H*-chromen-6-ol structure, 6-hydroxy-chromenols are derived from 2-methyl-2*H*-chromen-6-ol (**Figure 1**). The respective bicyclic core structure is associated to a side-chain with varying chain length and modifications, resulting in a great diversity of chromanol and chromenol derivates (Birringer et al., 2018). In a systematic review, Birringer and coworkers were the first implying the great potential of these structures by providing a comprehensive overview of the structural diversity and chemical transformation of all 230 chromanols and chromenols known at that time together with their natural source. The aim of the comprehensive review was rather the detailed description of the complexity of this group of compounds than an outline of their biological activity. Based on this systematic review, the intention of our review was to more selectively describe the effects of this class of natural products on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis, and the underlying molecular modes of action for selected chromanols and chromenols. Our review therefore represents a useful and relevant addition to the work of Birringer et al., focusing on the evaluation of selected compounds with known biological activity as possible lead structures for putative therapeutic approaches. Based on the mentioned inclusion criteria, we here focus on tocopherol (TOH) and tocotrienol (T3) structures, sargachromanols, amplexichromanols, and sargachromenols, which show structure-activity relationships with mostly anti-inflammatory as well as anti-carcinogenic activities.

Tocopherols and T3s differ in the saturation of the side-chain and form in its entirety the group of vitamin E. Based on the methylation pattern of the chromanol ring system α-, β-, γ-, δ-forms of TOHs and T3s can be distinguished. Oxidative modifications of the terminal side-chain increase anti-inflammatory activities. Therefore, hepatic metabolites of vitamin E are supposed to have important physiological activities and will also be included in this review. Sargachromanols (SCA), sargachromenols (SCE), and amplexichromanols (AC) have a tocotrienol-derived backbone implying similar biological activities. Our review focuses in more detail on the current knowledge about the biological activity as well as on potential regulatory pathways and molecular targets of chromanols and chromenols.

Abbreviations: α-T-13′-COOH, α-13′-carboxychromanol; α-T-13′-OH, α-13′-hydrochromanol; δ-T3-13′-COOH, garcinoic acid; AC, amplexichromanol; BMDM, bone marrow-derived macrophages; CEHC, carboxyethyl-hydroxychromanol; JNK, c-Jun N-terminal kinase; CoA, coenzyme A; COX, cyclooxygenases; CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; ICM, intermediate-chain metabolite; IL, interleukin; iNOS, inducible nitric oxide synthase; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; LCM, long-chain metabolite; LPS, lipopolysaccharide; LO, lipoxygenases; MAPKs, mitogen-activated kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; NO, nitric oxide; PARP-1, poly-[ADP-ribose]-polymerase 1; PG, prostaglandin; PMA, phorbol-12-myristat-13-acetate; PMNL, polymorphonuclear neutrophils; ROS, reactive oxygen species; SCA, sargachromanols; SCE, sargachromenols; SCM, short-chain metabolites; TX, thromboxane; TOH, tocopherol; TNF-α, tumor necrosis factor α; T3, tocotrienol.

CHROMANOL AND CHROMENOL STRUCTURES

Chromanols

Tocopherols and Tocotrienols

Vitamin E, more precisely *RRR*-α-tocopherol, has been identified in 1922 as a vital factor for fertility in rats (Evans and Bishop, 1922). Vitamin E does naturally occur in various plant-derived foods, such as oils, nuts, germs, seeds as well as vegetables and, in lower amounts, fruits. Thus, vitamin E represents the most widely distributed and abundant chromanol in nature. The term vitamin E comprises different lipophilic molecules that consist of the chromanol ring structure with a covalently bound phytyl-like side-chain. Depending on the saturation of the C-16′ side-chain, these molecules are classified as TOH, T3s (**Figure 2**), and vitamin E related structures named tocomonoenols and marine-derived TOHs. Tocopherols are characterized by a saturated phytyl side-chain whereas tocomonoenols, marine-derived TOHs and T3 are unsaturated at either the terminal isoprene unit or have three double bonds within the side-chain (Fujisawa et al., 2010; Kruk et al., 2011). Further, the methylation pattern of the chromanol ring determines the classification as α-, β-, γ-, and δ-forms of TOHs and T3s. Although several similar molecules form the group of vitamin E, only α-TOH seems to have vitamin property in animals and humans. For instance, in rats α-TOH preserves fertility, whereas in humans the deficiency disease *ataxia with vitamin E deficiency* (AVED) is prevented by α-TOH supplementation (Azzi, 2019).

For a long time, the health-promoting effects of vitamin E were only attributed to its antioxidant properties, but more recent studies revealed additional non-antioxidant functions of vitamin E. It is evident that vitamin E modulates gene expression and enzyme activities and also interferes with signaling cascades (Brigelius-Flohé, 2009; Zingg, 2019). Examples for these regulatory effects are the suppression of inflammatory mediators, reactive oxygen species (ROS) and adhesion molecules, the induction of scavenger receptors as well as the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (reviewed in Glauert, 2007; Rimbach et al., 2010; Wallert et al., 2014b; Zingg, 2019).

All forms of vitamin E undergo metabolic degradation in the liver. Although the detailed mechanisms remain poorly understood, the principles of the degradation of vitamin E to vitamer-specific physiological metabolites with intact chromanol ring (the nomenclature as α-, β-, γ- and δ-metabolites is used as described for the metabolic precursors in order to distinguish the

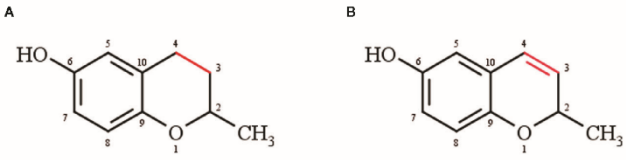
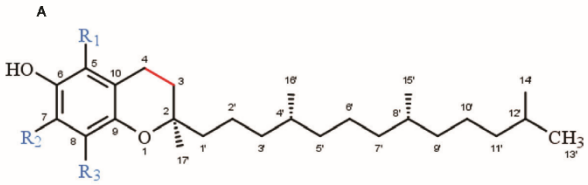
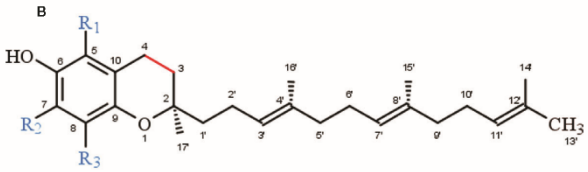


FIGURE 1 | **(A)** Chromanol (2-methyl-3,4-dihydro-2*H*-chromen-6-ol) and **(B)** chromenol (2-methyl-2*H*-chromen-6-ol) core structure.



-tocopherol	R_1	R_2	R_3
α	$-CH_3$	$-CH_3$	$-CH_3$
β	$-CH_3$	-H	$-CH_3$
γ	-H	$-CH_3$	$-CH_3$
δ	-H	-H	$-CH_3$

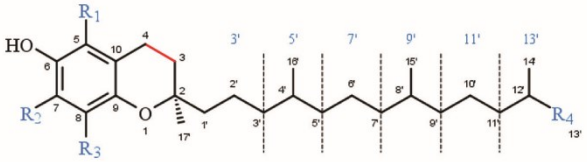


-tocotrienol	R_1	R_2	R_3
α	$-CH_3$	$-CH_3$	$-CH_3$
β	$-CH_3$	-H	$-CH_3$
γ	-H	$-CH_3$	$-CH_3$
δ	-H	-H	$-CH_3$

FIGURE 2 | Core structure of **(A)** tocopherol and **(B)** tocotrienol forms.

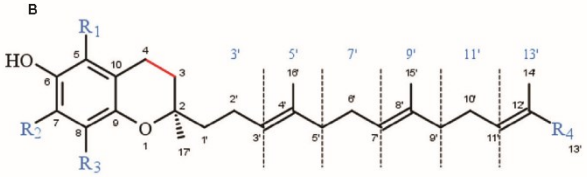
different forms of vitamin E metabolites) is widely accepted (**Figure 3**). Thus, enzymatic modifications are restricted to the side-chain (extensively reviewed in (Kluge et al., 2016; Schmölz et al., 2016)). α-Tocopherol is the main form of vitamin E in the human body due to its higher binding affinity to the α-tocopherol transfer protein (Hosomi et al., 1997). Thus, we will focus on the metabolic conversion of α-TOH in the following. Nevertheless, it should be noted that all forms of vitamin E (TOHs as well as T3s) follow the same metabolic route. However, due to the unsaturated side-chain, the degradation of T3s requires further enzymes such as 2,4 dienoyl-coenzyme A (CoA) reductase and 3,2-enoyl-CoA isomerase, which are also

A



α/β/γ/δ -metabolite	R_4	sidechain length
-13'-T-OH	-OH	13
-13'-T-COOH	-COOH	13
-11'-T-COOH	-COOH	11
-9'-T-COOH	-COOH	9
-7'-T-COOH	-COOH	7
-5'-T-COOH	-COOH	5
-3'-T-COOH	-COOH	3

B



α/β/γ/δ -metabolite	R_4	sidechain length
-13'-T3-OH	-OH	13
-13'-T3-COOH	-COOH	13
-11'-T3-COOH	-COOH	11
-9'-T3-COOH	-COOH	9
-7'-T3-COOH	-COOH	7
-5'-T3-COOH	-COOH	5
-3'-T3-COOH	-COOH	3

FIGURE 3 | Core structure of **(A)** tocopherol and **(B)** tocotrienol metabolites. The substitutions R1 to R3 are given in **Figure 2 (A)** and **(B)**.

involved in the metabolism of unsaturated fatty acids (Birringer et al., 2002). The initial step of α-TOH modification *via* ω-hydroxylation in the endoplasmic reticulum leads to the formation of the long-chain metabolite (LCM) α-13′-hydroxychromanol (α-T-13′-OH). It is supposed that this hydroxylation is catalyzed by cytochrome P450 (CYP)4F2 and CYP3A4 (Parker et al., 2000; Sontag and Parker, 2002). After its transfer from the endoplasmic reticulum to the peroxisome, α-T-13′-OH is converted to α-13′-carboxychromanol (α-T-13′-COOH) *via* ω-oxidation, likely *via* a two-step mechanism involving alcohol and aldehyde dehydrogenases. α-T-13′-OH and α-T-13′-COOH have been found in human serum (Wallert et al., 2014a; Ciffolilli et al., 2015; Giusepponi et al., 2017), supporting the idea of a more complex physiologic role of vitamin E with physiological relevance of its metabolites for various processes. In healthy humans α-TOH is the most abundant form of vitamin E, occurring in concentrations of about 20–30 μM in serum (Péter et al., 2015). However, supplementation of α-TOH increases α-TOH serum concentration in humans up to 90 μM (Dieber-Rotheneder

et al., 1991). Following supplementation, the hepatic metabolism is enhanced to protect the liver from excessive accumulation of α-TOH. Consequently, metabolites of vitamin E are formed and accumulate in turn in human serum. The LCMs α-T-13′-OH and α-T-13′-COOH were found in concentrations of 1–7 nM and 1–10 nM at baseline, respectively, whereas supplementation of α-TOH increased serum concentrations of the LCMs up to 12–32 nM and 3–55 nM, respectively (Wallert et al., 2014a; Ciffolilli et al., 2015; Giusepponi et al., 2017). Recent studies showed that the active metabolites of vitamin E exert effects on lipid metabolism, apoptosis, proliferation, and inflammatory processes as well as xenobiotic metabolism (Wallert et al., 2014a; Jang et al., 2016; Podszun et al., 2017; Schmölz et al., 2017). Finally, α-T-13′-COOH is excreted *via* bile and feces or is further degraded *via* several rounds of oxidation to the hydrophilic short-chain metabolite α-carboxyethyl-hydroxychromanol (CEHC), which is largely excreted *via* urine (Zhao et al., 2010; Johnson et al., 2012; Jiang, 2014). Another characteristic of the hepatic degradation of vitamin E is that the metabolites are chemically modified. In particular, the LCMs and the short-chain metabolites (SCMs) have been found as sulfated or glucuronidated conjugates in different biological matrices (Galli et al., 2002; Wallert et al., 2014a). Freiser and Jiang (2009) reported that more than 75% of γ-CEHC in the plasma of γ-T3-supplemented rats occurred in conjugated form. Further, also the LCMs, especially 13′-COOH and 11′-COOH metabolites were found as conjugates. Conjugation (sulfation or glucoronidation) seems to occur in the liver in parallel to the β-oxidation of the side-chain of vitamin E (Freiser and Jiang, 2009).

Beside the mentioned LCMs, intermediate-chain metabolites (ICMs) and SCMs that are formed *via* hepatic degradation of the different vitamin E forms, and vitamin E is also the precursor of quinones, representing another class of vitamin E-derived metabolites that exhibit antioxidant activity. Vitamin E quinones, in particular α-TOH-derived quinones, are formed as byproducts of α-TOH oxidation during peroxidation reactions in *in vitro* systems (Liebler et al., 1990; Infante, 1999). In addition, these metabolites can also be synthesized by photosynthetic organisms (Liebler et al., 1990). Although the knowledge on this group of tocopherol-derived metabolites is sparse, α-TOH quinone has been described as an essential enzymatic cofactor for fatty acid desaturase (Liebler et al., 1990).

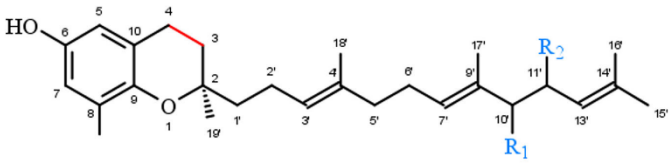
The natural compound δ-T3-13′-COOH, also known as δ-garcinoic acid or δ-tocotrienolic acid, shares structural similarity with the δ-T-LCM δ-T-13′-COOH, the second LCM originating from the hepatic metabolism of δ-TOH. As described previously, hepatic metabolism of tocotrienols follows that of tocopherols. Consequently, δ-T3-13′-COOH is formed during the degradation of δ-T3. Since the concentration of δ-T3 in human plasma is below 1% compared to α-TOH, the physiological relevance of δ-T3-13′-COOH in humans is likely low. So far, the detection of δ-T3-13′-COOH in human blood is still pending. However, local accumulation of δ-T3-13′-COOH in cells or tissues cannot be excluded. δ-T3-13′-COOH can be obtained in relatively high amounts and purity from the seeds of *Garcinia kola E. Heckel* (Bartolini et al., 2019; Wallert et al., 2019), a plant that is used in traditional African ethnomedicine (extensively reviewed in Kluge et al., 2016). This compound can be used as precursor for the semi-synthesis of α- and δ-LCMs (including α-T-13′-OH, α-T-13′-COOH, δ-T-13′-OH, and δ-T-13′-COOH) for experimental use *in vitro* and in mice and is therefore important for vitamin E metabolite research (Maloney and Hecht, 2005; Birringer et al., 2010). Further, δ-T3-13′-COOH also appeared to be a potent anti-inflammatory (Wallert et al., 2019) and anti-proliferative agent (Mazzini et al., 2009) and has been shown to act as an inhibitor of DNA polymerase β (Maloney and Hecht, 2005), indicating that δ-T3-13′-COOH may disturb base excision repair in tumor cells. A recent preprint of Bartolini et al. described δ-T3-13′-COOH as a potent agonist of PXR, which is known to be involved in inflammatory processes (Bartolini et al., 2019).

Sargachromanols

Sargachromanols (SCA) comprise a group of chromanols that occur in the brown algae family *Sargassaceae* (**Figure 4**). Their high structural diversity results from various side-chain modifications, leading to their classification from SCA-A to SCA-S. The entirety of sargachromanols has been isolated from *Sargassum siliquastrum* and has been classified *via* two-dimensional nuclear magnetic resonance experiments (Jang et al., 2005; Im Lee and Seo, 2011). The extensive analysis revealed detailed structural differences between the sargachromanols. For example SCA-C contains a 9′-hydroxyl group with *R*-configuration in the side-chain, while SCA-F has a methoxy group at C-9′ and a hydroxyl group with *R*-configuration at C-10′ (extensively reviewed in Birringer et al., 2018). SCAs have been reported to exhibit various biological activities, including anti-oxidative (Lim et al., 2019) (SCA-G), anti-osteoclastogenic (Yoon et al., 2012b; Yoon et al., 2013) (SCA-G), anti-inflammatory (Yoon et al., 2012a; Lee et al., 2013; Heo et al., 2014) (SCA-G and SCA-D), as well as anti-diabetic (Pak et al., 2015) (SCA-I) ones. To the best of our knowledge, metabolism of sargachromanols in humans or animals has not been investigated.

Amplexichromanols

Amplexichromanols represent a small group of hydroxylated T3 derivatives found in different parts of *Garcinia* plants. For instance, lipophilic extracts from the bark of *Garcinia amplexicaulis* were used to isolate γ-AC and δ-AC (**Figure 5**). The chemical structure of γ-AC and δ-AC are similar to γ-T3 and δ-T3, respectively, but carry two additional hydroxyl groups at C-13′ and C-14′. In an initial *in vitro* experiment, δ-AC reduced vascular endothelial growth factor induced cell proliferation in low nanomolar concentrations, while γ-AC had no effect. This observation probably indicates distinct efficiencies for the different amplexichromanols (Lavaud et al., 2013). However, further experiments revealed strong anti-oxidative potential for both compounds (Lavaud et al., 2015), but nothing is known about the metabolization, systemic distribution, tissue accumulation, or excretion of amplexichromanols so far.

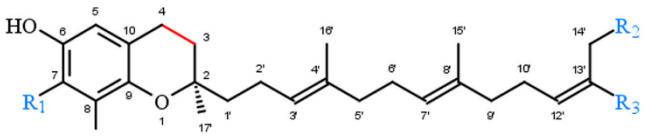


Sargachromanol	R_1	R_2
D	-OH (R)	-OH (S)
E	-OH (R)	-OH (R)
F	-OMe	-OH (S)
G	=O	-OH (S)

FIGURE 4 | Core structure of sargachromanol forms.

Chromenols

Chromenols consist of a 2-methyl-2*H*-chromen-6-ol core that is associated with a side-chain with varying chain length and varying chemical modifications, leading to high structural diversity. The multitude of these compounds can be obtained from photosynthetic organisms like plants, algae, cyanobacteria, fungi, corals, sponges, and tunicates (Birringer et al., 2018). As the current knowledge on the biological functions of chromenol structures is sparse, this review will exemplarily focus on the most studied sargachromenols (**Figure 6**). Similar to their chromanol counterparts, sargachromenols were named after the brown algae species *Sargassum serratifolium*, from which they have been isolated first (Kusumi et al., 1979). Just like sargachromanols, sargachromenols comprise a molecule class of high structural diversity due to different side-chain modifications. In the first systematic review on the field of chromanols and chromenols, Birringer and coworkers described 15 sargachromenols, 13 compounds with marine origin (brown algae) and two with marine and plant origin (Birringer et al., 2018). As an example, δ-SCE, a structure consisting of a δ-chromenol ring system with an unsaturated side-chain containing a carboxy group at C-15′, is widely distributed in algae of the *Sargassaceae* family but can also be obtained from plants like *Iryanthera juruensis*. Another interesting sargachromenol is dehydro-δ-T3, or Sargol, which is supposed to serve as a biosynthetic precursor for most of the sargachromenols and is occurring in brown algae (Birringer et al., 2018). Brown algae from the *Sargassaceae* family have been used in traditional Asian medicine as well as in health promoting diets, revealing a variety of biological functions (Kim et al., 2014). For example, ethanolic extracts from the *Sargassaceae* species *Myagropsis myagroides*, an alga that grows at the coast of East Asia, revealed potent anti-inflammatory activity. After HPLC-based separation, sargachromenols



-amplexichromanol	R_1	R_2	R_3
γ	$-CH_3$	-OH	$-CH_2OH$
δ	-H	-OH	$-CH_2OH$

FIGURE 5 | Core structure of amplexichromanol forms.

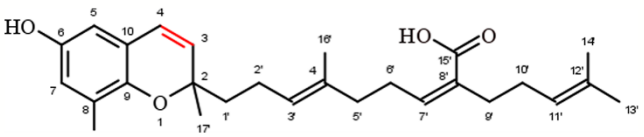


FIGURE 6 | Molecular structure of δ-sargachromenol.

(mostly δ-SCE) have been identified as the most potent anti-inflammatory compounds within these extracts, based on their inhibitory effect on nitric oxide (NO) production in lipopolysaccharide (LPS)-treated immortalized murine microglial BV-2 cells (Kim et al., 2014). Beside their anti-inflammatory activity, anti-carcinogenic (Hur et al., 2008), anti-photoaging (Kim et al., 2012), and anti-cholinesterase activities (Choi et al., 2007) have been described for SCEs. Further, sargachromenols isolated from *Sargassum macrocarpum* mediate nerve-growth-factor-driven neuronal growth in pheochromocytoma of rat adrenal medulla derived PC12D cells (Tsang et al., 2005).

BIOLOGICAL ACTIVITY OF NATURAL CHROMANOLS AND CHROMENOLS

Based on published data, we have chosen signaling pathways that are central for inflammation, apoptosis, cell proliferation, and carcinogenesis (**Figure 7**). Respective effects of tocopherol-

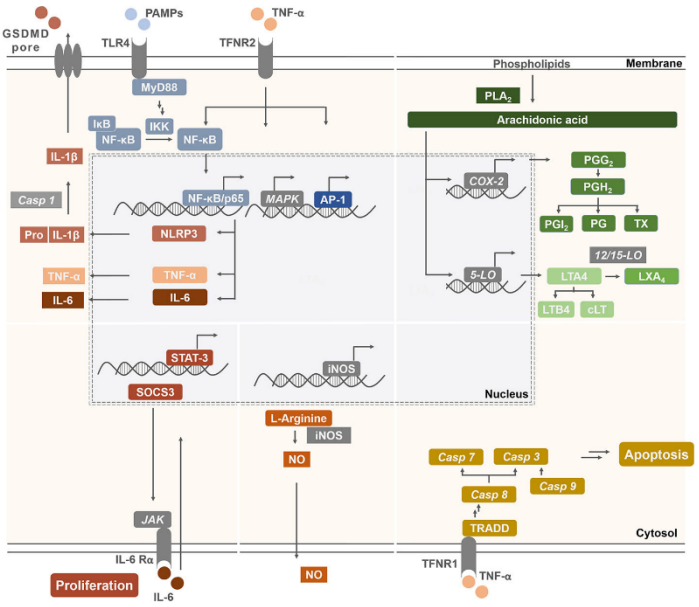


FIGURE 7 | Schematic illustration of signaling targets, pathways, and molecules involved in inflammatory response and cancer progression. Pathways were chosen due to known interactions with the compounds of interest. Inflammatory signaling molecules are interleukins (IL), tumor necrosis factor-α (TNF-α), nitric oxide (NO), prostaglandins (PG), prostacyclin (PGI_2), tromboxanes (TX), leukotriens (LT), and lipoxins (LX). Their expression, synthesis, or release depends, among others, on the activation of NF-κB, NLR family pyrin domain containing 3 (NLRP3) inflammasome, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and lipoxygenases (LO). In addition, membrane receptors, such as cytokine or epidermal growth factor receptors regulate the activation of nuclear receptor signal transducer and activator of transcription (STAT) 3 as well as extrinsic or intrinsic pathways which trigger the activation of caspases (Casp). These mediators are therefore important factors for modulating the balance between cell proliferation and apoptosis, which is essential to prevent carcinogenesis.

derived (T) and tocotrienol-derived (T3) chromanol and chromenol structures on nuclear receptors and target enzymes were screened and are discussed in the following.

Inflammation

Inflammation is essential for wound healing as well as defense and clearance of pathogens (Kunnumakkara et al., 2018). However, excessive and persistent inflammation is a driving force for many chronic diseases. In addition to obvious inflammatory diseases such as rheumatoid arthritis, it is well accepted that cancer, Alzheimer's disease, and metabolic syndrome-related diseases like atherosclerosis, non-alcoholic fat liver disease, and diabetes mellitus type 2 are triggered by chronic low-grade inflammation (Kunnumakkara et al., 2018). As systemic inflammation is a complex process, this review refers only to inflammatory pathways that have been studied for chromanol and/or chromenol structures. Key regulatory factors and mediators of inflammatory processes in this context are receptors that sense proinflammatory stimuli, e.g. the toll-like receptors (TLRs), intracellular signaling molecules, like mitogen-activated protein kinases (MAPKs), and transcription factors, such as NF-κB or nuclear factor erythroid 2-related factor 2 (Nrf2). Further, enzymes that produce pro-inflammatory mediators such as prostaglandins (PGs) and leukotrienes (LTs) play a central role during the coordinated orchestra of the inflammatory process. This includes cyclooxygenases (COX) and lipoxygenases (LO). Other key players of inflammation are cytokines which are secreted by various cells and affect the interaction and communication between the different types of cells involved in inflammation (Aggarwal, 2009; Kunnumakkara et al., 2018). Important pro-inflammatory cytokines are interleukin (IL)-1β, IL-6, and IL-8 as well as tumor necrosis factor-α (TNF-α). Another important signaling molecule in inflammatory processes is nitric oxide (Aggarwal, 2009). In the following, chromanol and chromenol structures regulating the expression of key pro-inflammatory enzymes and the respective formation of signaling molecules are outlined.

Chromanols

A detailed overview on the biological activities of chromanols linked to inflammation is provided in **Table 1**.

Tocopherols and Tocotrienols

Data available for TOHs and T3s correlate with their abundance in humans. Therefore, α- and γ-TOH as well as their respective T3 forms were mostly investigated so far. α-Tocopherol is regarded as the only form within the group of vitamin E that has been shown to mediate actual vitamin E function (Azzi, 2019). Further, α-TOH is considered as the most abundant vitamin E form in human nutrition, followed by γ-TOH. Relevance of T3s as anti-inflammatory compounds has just recently come to fore of research and will be presented in the following sections.

Tocopherols. For many years, TOHs were solely known for their anti-oxidative capacity. However, Azzi and colleagues discovered additional gene regulatory effects of α-TOH that are independent from its capacity as an antioxidant. α-TOH revealed distinct effects on nitric oxide- and eicosanoid-mediated inflammation. For instance, α-TOH (10 μM) decreased the expression level of inducible nitric oxide synthase (iNOS) in LPS-stimulated murine RAW264.7 macrophages (Jiang et al., 2000). However, others could not confirm the observed alteration of iNOS expression using 5 μM (Wallert et al., 2015), 20 μM (Schmölz et al., 2017), or even 100 μM (Ciffolilli et al., 2015) α-TOH. In line with this, iNOS-mediated formation of nitric oxide remained unchanged in RAW264.7 macrophages by coincubation with α-TOH (Jiang et al., 2000; Ciffolilli et al., 2015; Wallert et al., 2015). In contrast, the formation of PGE_2 was blocked by 23 to 100 μM α-TOH in LPS-stimulated RAW264.7 macrophages (Jiang et al., 2000; Yam et al., 2009; Ciffolilli et al., 2015; Wallert et al., 2015), but not in IL-1β-stimulated A549 epithelial cells (Jiang et al., 2008). Unexpectedly, upstream-regulated COX-2 expression and activity remained unchanged in RAW264.7 macrophages at concentrations of 23 to 100 μM α-TOH. Furthermore, cytokine-mediated inflammation was not regulated by α-TOH (Yam et al., 2009), except for an inhibition of IL-1β gene expression in RAW264.7 macrophages using 100 μM (Ciffolilli et al., 2015; Wallert et al., 2015). Beside external stimuli, induction of inflammation, mainly *via* the TLR4-NF-κB signaling pathway, senescence of cells, and aging are also known triggers of inflammation (Lasry and Ben-Neriah, 2015). Indeed, 24-months-old mice are characterized by an increased inflammatory state compared to younger mice (six months). Application of 500 ppm α-TOH acetate lowered aging-induced increases of nitric oxide and PGE_2 plasma levels as well as COX-2 activity compared to 24-months-old mice fed 30 ppm (Beharka et al., 2002). In line with this, supplementation with 800 mg α-TOH/kg/d in elder humans for 30 days lead to significantly lower levels of PGE_2 in plasma and peripheral blood mononuclear cells compared to vehicle-treated controls (Meydani et al., 1990).

The second most abundant form of vitamin E, γ-TOH, is more prominent for its anti-inflammatory capacity compared to α-TOH. Release of nitric oxide by LPS-stimulated RAW264.7 cells was significantly inhibited using 10 μM γ-TOH (Jiang et al., 2000). Release of eicosanoids inflammation, more precisely PGE_2, in LPS-stimulated RAW264.7 cells and in IL-1β-stimulated A549 cells was inhibited by 10 μM (IC_{50} 7.5 μM) (Jiang et al., 2000) and 25–40 μM (IC_{50} 4–7 μM) (Jiang et al., 2000; Jiang et al., 2008), respectively. However, COX-2 expression (Jiang et al., 2000; Jiang et al., 2008) and activity (Jiang et al., 2000; Jang et al., 2016) remained unchanged in LPS-stimulated RAW264.7 macrophages, whereas COX-2 activity was inhibited by 50 μM γ-TOH in IL-1β-stimulated A549 epithelial cells (Jiang et al., 2008). Azoxymethane-induced IL-6 production was dampened in BALB/c mice by a γ-TOH-enriched diet (Jiang et al., 2013).

δ-tocopherol (20 μM) significantly decreased LPS-induced expression of iNOS (by 60% at mRNA and by 48% at protein level) and formation of nitric oxide (by 36%) in RAW264.7 macrophages (Schmölz et al., 2017). Jiang et al. reported an inhibition of COX-2 activity, but not COX-2 expression in IL-1β-stimulated A549 cells (Jiang et al., 2008), whereas Jang et al.

TABLE 1 | Overview on the biological activities of chromanols linked to inflammation.

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
α-TOH								
LPS iNOS PE RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS NO PrD RAW264.7 20 μM no inhibition (Wallert et al., 2015)	LPS COX-2 PE, A RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS COX-2 GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS PGE_2 PrD RAW264.7 100 μM inhibition (Wallert et al., 2015)	IL-1β PGE_2 PrD A549 cells 50 μM no inhibition (Jiang et al., 2008)	LPS pro IL-1β GE RAW264.7 100 μM inhibition (Wallert et al., 2015)	LPS IL-6 GE RAW264.7 100 μM no inhibition (Wallert et al., 2015)	LPS TNF-α GE RAW264.7 100 μM no inhibition (Wallert et al., 2015)
LPS iNOS PE RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS NO PrD RAW264.7 10 μM no inhibition (Jiang et al., 2000)	LPS COX E, A RAW264.7 50 μM no inhibition (Jiang et al., 2000)	LPS COX-2 A m_PM 500 ppm inhibition (Beharka et al., 2002)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Jiang et al., 2000)	Age PGE_2 PrD human 800 mg/d inhibition (Meydani et al., 1990)	LPS pro IL-1β GE RAW264.7 100 μM inhibition (Ciffolilli et al., 2015)	LPS IL-6 PrD RAW264.7 23 μM no inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 23 μM induction (Yam et al., 2009)
LPS iNOS E RAW264.7 20 μM no inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 20 μM induction (Schmölz et al., 2017)	LPS COX-2 E RAW264.7 23 μM no inhibition (Yam et al., 2009)		LPS PGE_2 PrD RAW264.7 23 μM induction (Yam et al., 2009)	LPS PGE_2 PrD RAW264.7 100 μM inhibition (Ciffolilli et al., 2015)		LPS IL-6 GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS TNF-α GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)
LPS iNOS GE RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)	LPS NO PrD RAW264.7 100 μM no inhibition (Ciffolilli et al., 2015)			LPS PGE_2 PrD m_PM 500 ppm inhibition (Beharka et al., 2002)				
	LPS NO PrD m_PM 500 ppm inhibition (Beharka et al., 2002)							
β-TOH								
				IL-1β PGE_2 PrD A549 cells 50 μM no inhibition (Jiang et al., 2008)				
γ-TOH								
LPS iNOS PE RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS NO PrD RAW264.7 10 μM inhibition (Jiang et al., 2000)	LPS COX E + A RAW.264.7 50 μM no inhibition (Jiang et al., 2000)	IL-1β COX-2 A A549 cells 50 μM inhibition (Jiang et al., 2008)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 cells 25 μM inhibition (Jiang et al., 2008)		AOM IL-6 PrD BALB/c mice 0.1% of diet inhibition (Jiang et al., 2013)	
		IL-1β COX-2 PE A549 cells 40 μM no inhibition (Jiang et al., 2008)	LPS COX A A549 10 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 cells 40 μM inhibition (Jiang et al., 2000)				

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
δ-TOH								
LPS iNOS E RAW264.7 20 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 20 μM inhibition (Schmölz et al., 2017)	IL-1β COX-2 PE A549 cells 40 μM no inhibition (Jiang et al., 2008)	IL-1β COX-2 A A549 cells 50 μM inhibition (Jiang et al., 2008)	IL-1β PGE_2 PrD A549 cells 25 μM inhibition (Jiang et al., 2008)				
α-T3								
	LPS NO PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)	IL-1β COX-2 PE A549 cells 10 μM no inhibition (Jiang et al., 2008)	LPS COX-2 PE RAW264.7 23.5 μM no inhibition (Yam et al., 2009)				LPS IL-6 PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 23.5 μM inhibition (Yam et al., 2009)
		LPS COX-2 GE RAW264.7 23.5 μM inhibition (Yam et al., 2009)						
γ-T3								
	LPS NO PrD RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS COX-2 GE RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS COX-2 GE m_BMDM 1 μM inhibition (Kim et al., 2018)	LPS PGE_2 PrD RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS PGE_2 PrD m_BMDM 1 μM inhibition (Kim et al., 2018)	LPS pro IL-1β GE/PrD m_BMDM 1 μM inhibition (Kim et al., 2018)	LPS IL-6 PrD RAW264.7 24 μM inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 24 μM no inhibition (Yam et al., 2009)
		LPS COX-2 PE RAW264.7 24 μM no inhibition (Yam et al., 2009)				LPS pro IL-1β PrD m_BMDM 1 μM inhibition (Kim et al., 2016)	diabetes IL-6 PrD *db/db* mice 0.1% of diet inhibition (Kim et al., 2016)	diabetes TNF-α PrD *db/db* mice 0.1% of diet inhibition (Kim et al., 2016)
						LPS IL-1β PrD m_BMDM 1 μM inhibition (Kim et al., 2016)		
δ-T3								
	LPS NO PrD RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS COX-2 GE RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS COX-2 PE RAW264.7 25 μM no inhibition (Yam et al., 2009)	LPS PGE_2 PrD RAW264.7 25 μM inhibition (Yam et al., 2009)			LPS IL-6 PrD RAW264.7 25 μM inhibition (Yam et al., 2009)	LPS TNF-α PrD RAW264.7 25 μM induction (Yam et al., 2009)
α-T-13′-OH								
LPS iNOS E RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS NO PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS COX-2 E RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)		LPS PGE_2 PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)		LPS IL-1β PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS IL-6 PrD RAW264.7 10 μM inhibition (Ciffolilli et al., 2015)	LPS TNF-α PrD RAW264.7 10 μM no inhibition (Ciffolilli et al., 2015)

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
LPS iNOS E RAW264.7 10 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 10 μM inhibition (Schmölz et al., 2017)							
α-T-13′-COOH								
LPS iNOS PE RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS NO PrD RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS COX-2 PE RAW264.7 5 μM inhibition (Wallert et al., 2015)	IL-1β COX-2 A platelet 10 μM inhibition (Pein et al., 2018)	LPS PGE_2 PrD RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS PGE_2 PrD h_monocytes 10 μM no inhibition (Pein et al., 2018)	LPS pro IL-1β GE RAW264.7 5 μM inhibition (Wallert et al., 2015)	LPS IL-6 GE RAW264.7 5 μM no inhibition (Wallert et al., 2015)	LPS TNF-α GE RAW264.7 5 μM no inhibition (Wallert et al., 2015)
LPS iNOS E RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS COX-2 A RAW264.7 5 μM no inhibition (Wallert et al., 2015)	– COX-2 A enzyme 10 μM no inhibition (Pein et al., 2018)					
δ-T-13′-OH								
LPS iNOS E RAW264.7 10 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 10 μM inhibition (Schmölz et al., 2017)							
δ-T-13′-COOH								
LPS iNOS E RAW264.7 5 μM inhibition (Schmölz et al., 2017)	LPS NO PrD RAW264.7 5 μM inhibition (Schmölz et al., 2017)	IL-1β COX-2 A A549 4 μM inhibition (Jiang et al., 2008)	– COX-2 A enzyme 5 μM inhibition (Jiang et al., 2016)					
		– COX-2 A enzyme 4 μM inhibition (Jiang et al., 2008)						
δ-T-9′-COOH								
		– COX-2 A enzyme 20 μM no inhibition (Jiang et al., 2008)	IL-1β COX-2 A A549 6 μM inhibition (Jiang et al., 2008)					
α-T-5′-COOH								
		– COX-2 A enzyme 140 μM inhibition (Jiang et al., 2008)						

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated				Cytokine-mediated		
iNOS	**NO**	**COX-2**		**PGE_2**		**IL-1β**	**IL-6**	**TNF-α**
α-T-3′-COOH								
TNF-α iNOS PE EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	TNF-α NO PrD RAEC cells 100 μM inhibition (Grammas et al., 2004) TNF-α/LPS NO PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)			LPS PGE_2 PrD RAEC cells 100 μM inhibition (Grammas et al., 2004)	LPS PGE_2 PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)			
γ-T-3′-COOH								
TNF-α iNOS E EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	LPS NO PrD RAW264.7 10 μM no inhibition (Jiang et al., 2000) LPS NO PrD EOC-20 cells 100 μM inhibition (Grammas et al., 2004)	IL-1β COX-2 A A549 50 μM inhibition (Jiang et al., 2000) IL-1β COX-2 PE A549 50 μM no inhibition (Jiang et al., 2000)	– COX-2 A enzyme 450 μM inhibition (Jiang et al., 2008)	LPS PGE_2 PrD RAW264.7 10 μM inhibition (Jiang et al., 2000)	IL-1β PGE_2 PrD A549 40 μM inhibition (Jiang et al., 2000)			
δ-T3-13′-COOH								
LPS iNOS E RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS NO PrD RAW264.7 5 μM inhibition (Wallert et al., 2019)	– COX-2 A enzyme 9.8 μM inhibition (Jang et al., 2016) LPS COX-2 E RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS COX-2 A h_monocytes 10 μM no inhibition (Pein et al., 2018)	LPS PGE_2 PrD RAW264.7 5 μM inhibition (Wallert et al., 2019) HFD PGE_2 PrD m_APOE$^{-/-}$ 1 mg/kg no inhibition (Wallert et al., 2019)	LPS PGE_2 PrD h_monocytes 300 nM inhibition (Pein et al., 2018)	LPS pro IL-1β GE RAW264.7 5 μM inhibition (Wallert et al., 2019) HFD IL-1β PrD m_APOE$^{-/-}$ 1 mg/kg no inhibition (Wallert et al., 2019)	LPS IL-6 GE RAW264.7 5 μM inhibition (Wallert et al., 2019)	LPS TNF-α GE RAW264.7 5 μM inhibition (Wallert et al., 2019)
SCA D								
LPS iNOS PE RAW264.7 15 μM inhibition (Heo et al., 2014)	LPS NO PrD RAW264.7 15 μM inhibition (Heo et al., 2014)	LPS COX-2 PE RAW264.7 15 μM inhibition (Heo et al., 2014)		LPS PGE_2 PrD RAW264.7 15 μM inhibition (Heo et al., 2014)		LPS IL-1β PrD RAW264.7 60 μM inhibition (Heo et al., 2014)	LPS IL-6 PrD RAW264.7 30 μM inhibition (Heo et al., 2014)	LPS TNF-α PrD RAW264.7 60 μM inhibition (Heo et al., 2014)
SCA E								
LPS iNOS PE RAW264.7 29 μM inhibition (Lee et al., 2013)	LPS NO PrD RAW264.7 29 μM inhibition (Lee et al., 2013)	LPS COX-2 PE RAW264.7 29 μM inhibition (Lee et al., 2013)		LPS PGE_2 PrD RAW264.7 29 μM inhibition (Lee et al., 2013)		LPS IL-1β PrD RAW264.7 12 μM inhibition (Lee et al., 2013)		LPS TNF-α PrD RAW264.7 29 μM inhibition (Lee et al., 2013)

(Continued)

TABLE 1 | Continued

Nitric oxide		Eicosanoid-mediated		Cytokine-mediated		
iNOS	**NO**	**COX-2**	**PGE_2**	**IL-1β**	**IL-6**	**TNF-α**
SCA G						
LPS iNOS PE RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS NO PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS COX-2 PE RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS PGE_2 PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS IL-1β PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS IL-6 PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)	LPS TNF-α PrD RAW264.7 10 μM inhibition (Yoon et al., 2012a)
δ-AC						
				LPS IL-1β PrD monocytes 1 μM inhibition (Richomme et al., 2017)		LPS TNF-α PrD monocytes 10 μM inhibition (Richomme et al., 2017)

The effects of the respective compounds on inflammation have been divided into activities mediated by nitric oxide (iNOS, NO), eicosanoids (COX-2, PGE_2), and cytokines (IL-1β, IL-6, TNF-α). The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type, tissue, mouse, or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. When no stimulus was used or was required for the studies, the respective row is marked with a dash. The following abbreviations are used: A, activity; A549, human adenocarcinoma alveolar basal epithelial cells; BALB/c mice, albino laboratory-bred strain of the house mouse; Apoe$^{-/-}$ mice, apolipoprotein E deficient mice; BMDM, bone marrow derived macrophages; COX-2, cyclooxygenase 2; EOC-20, epithelial ovarian cancer cells; E, expression; GE, gene expression; HFD, high-fat diet; h, human; iNOS, inducible nitric oxide synthase; IL, interleukin; db/db mice, leptin receptor activity deficient mice; LPS, lipopolysaccharides; m, murine; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; NO, nitric oxide; ppm, parts per million; PM, peritoneal macrophages; PrD, production; PGE_2, prostaglandin E_2; PE, protein expression; RAEC, rat aortic endothelial cells; TNF-α, tumor necrosis factor α.
All results obtained from in vivo studies are marked in gray.

did not observe altered COX-2 activity after δ-TOH treatment using a human recombinant enzyme-based assay (Jang et al., 2016). However, formation of PGE_2 was significantly blocked (IC_{50} 1–3 μM) (Jiang et al., 2008). The least abundant form of tocopherols, β-TOH has been rarely studied regarding its anti-inflammatory capacity. Studies available so far did not reveal any anti-inflammatory effects of β-TOH (Jiang et al., 2008).

Tocotrienols. Recent publications reported a more pronounced anti-inflammatory capacity of T3s compared to TOHs, with γ-T3 and α-T3 showing the strongest effects. α-, δ-, and γ-T3 significantly decreased LPS-mediated formation of nitric oxide (by 11%, 31%, 19%, respectively) and PGE_2 (by 30%, 55%, 20%, respectively) in RAW264.7 macrophages treated with 23.5 μM of the respective compound (Yam et al., 2009) as well as bone marrow-derived macrophages (BMDMs) using 1 μM of γ-T3 (Kim et al., 2018). Expression of COX-2 mRNA was inhibited by α-, δ-, and γ-T3, whereas protein expression remained unchanged (Jiang et al., 2008; Yam et al., 2009; Kim et al., 2018). In addition, cytokine-driven inflammation is also dampened by α-, δ-, and γ-T3, which reduced the release of IL-6 and TNF-α in LPS-stimulated RAW264.7 cells. However, γ-T3 reduced expression of IL-6 and TNF-α mRNA as well as the secretion of IL-6, but not of TNF-α in this cell model (Yam et al., 2009). Furthermore, first reports suggest inhibitory effects of γ-T3 on the NLR family pyrin domain containing 3 (NLRP3) inflammasome. In brief, 1 μM γ-T3 suppressed mRNA expression of pro-IL-1β and -18 as well as respective formation of active IL-1β and -18. This has been observed in LPS/nigericin- as well as LPS/palmitate-stimulated BMDMs and *db/db* mice fed with a diet containing 0.1% γ-T3 for eight weeks (Kim et al., 2016; Kim et al., 2018).

Metabolites of Tocopherols and Tocotrienols

We here present a report on selected structures formed during hepatic catabolism of vitamin E, for which data on the biological activity was available. Metabolites formed during physiological hepatic metabolism of vitamin E are highly potent anti-inflammatory compounds with different efficiencies, depending on their methylation pattern (Azzi, 2019) and the number of isoprene units forming the side-chain (Schmölz et al., 2017). Metabolism of non-α-TOH forms of vitamin E is more pronounced, resulting from the lower affinities of these molecules to the α-tocopherol transfer protein. However, α-metabolites revealed significant anti-inflammatory properties. The most widely studied metabolites are the LCMs α-T-13′-OH and -COOH and the short-chain metabolites α- and γ-3′-T-COOH, likely due to their presence in plasma, feces, and urine, respectively, which may account for their physiological relevance (Jiang et al., 2007).

Long- and Intermediate-Chain Tocopherol-Derived Metabolites. Birringer and coworkers showed the relevance of the terminal oxidative modification of the side-chain for biological activity (Birringer et al., 2018). During the hepatic metabolism of TOHs, T-13′-OH are the first metabolites that are formed; these LCMs show distinct effects that are different from those of their respective metabolic precursor (for details, see **Chapter 2.1.1. *Tocopherols and Tocotrienols***). Both, α- and δ-T-13′-OH significantly decreased mRNA (29–72% and 87%, respectively) and

protein (40–53% and 53%, respectively) expression of iNOS and the production of nitric oxide (56–69% and 49%, respectively) in LPS-stimulated murine RAW264.7 macrophages at a concentration of 10 μM, thus showing comparable effect sizes independent from the methylation pattern of the chromanol ring system (Ciffolilli et al., 2015; Schmölz et al., 2017). Furthermore, α-T-13′-OH significantly decreased expression of COX-2 mRNA and protein (64% and 49%, respectively), IL-1β (64%) and IL-6 (68%) mRNA, and the production of PGE_2 (55%) (Ciffolilli et al., 2015).

Notably, the length of the side-chain is important for the mediation of anti-inflammatory effects. Accordingly, both α-T-13′-COOH (5 μM) and δ-T-13′-COOH (5 μM) significantly decreased expressions of iNOS and COX-2 mRNAs as well as proteins in murine LPS-stimulated RAW264.7 macrophages (Wallert et al., 2015; Schmölz et al., 2017). Further, δ-T-13′-COOH inhibited the activity of purified recombinant COX-2 enzyme (5 μM [Jiang et al., 2008; Jang et al., 2016]) as well as in human lung adenocarcinoma A549 cells (4 μM, [Jiang et al., 2008]). Interestingly, the activity of recombinant COX-2 enzymes remained unchanged by α-T-13′-COOH (5–10 μM) (Wallert et al., 2015; Pein et al., 2018). LPS-induced production of the respective signaling molecules, nitric oxide and PGE_2, was completely blocked in murine macrophages (5 μM), but not in LPS-activated human primary monocytes (10 μM) (Pein et al., 2018). In addition, 5-LO-induced formation of pro-inflammatory leukotrienes was dampened by α-T-13′-COOH in LPS-stimulated monocytes (LTB_4), activated human neutrophils, activated human blood, zymosan-induced mouse peritonitis (LTC4), as measured in plasma and exudate, and ovalbumin-induced bronchial hyperreactivity in mice (Pein et al., 2018). Effective concentrations of α-T-13′-COOH, that inhibit 5-LO product formation *in vitro*, were in a range that was detected for the metabolite in human and mice serum without supplementation (<0.3 μM). Furthermore, expression of pro-IL-1β was down-regulated by 5 μM α-T-13′-COOH, whereas IL-6 and TNF-α remained unchanged (Wallert et al., 2015).

Degradation of the LCMs of different vitamin E forms results in formation of respective ICMs that are further processed to SCMs. These metabolic end-products do not accumulate in plasma or tissues and their physiological relevance is therefore considered as less important. Hence, data on these metabolites are scarce. To date, anti-inflammatory effects, *i.e.* the inhibition of COX-2 activity (IC_{50} 6 μM), by δ-9′-T-COOH have been reported in human lung adenocarcinoma A549 cells (Jiang et al., 2008).

Long- and Intermediate-Chain Tocotrienol-Derived Metabolites. Within the group of T3-derived metabolites, the LCM δ-T3-13′-COOH (*i.e.* garcinoic acid) is the most potent anti-inflammatory compound of the ones studied so far. Expression of iNOS (by 97%), COX-2 (by 70%), pro-IL-1β (by 61%), IL-6 (by 70%), and TNF-α (by 25%) mRNA was decreased by 5 μM δ-T3-13′-COOH in LPS-stimulated murine RAW264.7 macrophages. Consequently, protein expression of iNOS (by 83%), COX-2 (by 33%), and the respective formation of NO (by 81%), PGE_2 (by 90%) and thromboxane $(TX)B_2$ (by 91%) were dampened in LPS-stimulated murine RAW264.7 macrophages (Wallert et al., 2019). Formation of PGE_2 in LPS-stimulated monocytes was inhibited already by 300 nM δ-T3-13′-COOH (Pein et al., 2018). In line with this, δ-T3-13′-COOH also inhibited activity of microsomal PGE_2 synthase (by nearly 70%) at a concentration of 10 μM in a cell-free assay using microsomes of IL-1β-stimulated human lung adenocarcinoma A549 cells as an enzyme source (Alsabil et al., 2016; Pein et al., 2018). However, in *Apoe*$^{-/-}$ mice fed a high fat diet with 1 mg/kg δ-T3-13′-COOH for 8 weeks neither nitric oxide, PGE_2, TXB_2 nor IL-1β concentrations in plasma were altered compared to the control group (Wallert et al., 2019). However, contrary data exist also for the alteration of prostaglandins following inhibition of COX-2 activity: IC_{50} 9.8 μM (Jang et al., 2016) and IC_{50} >10 μM (Pein et al., 2018).

Short-Chain Tocopherol-Derived Metabolites. 5′-T-COOH (CMBHC) and 3′-T-COOH (CEHC) are the SCMs. Physiologically formed γ-3′-T-COOH was mainly detected in urine. Supplementation of α-TOH enhances the hepatic metabolism of α-TOH, which in turn increases degradation of α-TOH and excretion of α-5′-T-COOH and α-3′-T-COOH *via* urine. Both, α-5′-T-COOH (IC_{50} 140 μM) and γ-3′-T-COOH (IC_{50} 450 μM) showed marginal inhibitory effects on human recombinant COX-2 activity (Jiang et al., 2008). However, in IL-1β-stimulated A549 cells, γ-3′-T-COOH (50 μM) exhibited stronger inhibition of COX-2 activity. Formation of PGE_2 was also inhibited in IL-1β-stimulated A549 (50 μM), LPS-stimulated RAW264.7 (10 μM), as well as TNF-α-stimulated RAEC (IC_{50} 59 μM) and EOC-20 cells (IC_{50} 66 μM) (Jiang et al., 2000; Grammas et al., 2004). The TNF-α-induced release of nitric oxide was blocked in EOC-20 (IC_{50} 58 μM) and RAEC cells (IC_{50} 56 μM) by α-3′-T-COOH, whereas 100 μM γ-3′-T-COOH inhibited production of nitric oxide in EOC-20 cells by 10% only (Grammas et al., 2004). In contrast, both α-3′-T-COOH and γ-3′-T-COOH decreased production of nitric oxide in LPS-stimulated EOC-20 cells (Grammas et al., 2004). Notably, lower concentrations did not alter production of nitric oxide (Jiang et al., 2000; Grammas et al., 2004).

Sargachromanols

The sargachromanol forms D, E, and G isolated from *Sargassum siliquastrum* also exert anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages in a concentration-dependent manner. Sargachromanol forms D, E, and G inhibited expression of iNOS protein to 30–50% with concentrations of 15, 12.5, and 20 μM, respectively. In contrast, inhibitory effects on the formation of the respective signaling molecule nitric varies compound-dependent between 10 and 90% (Lee et al., 2013), with SCA E being the most effective (Yoon et al., 2012a; Lee et al., 2013; Heo et al., 2014). Within the inflammatory eicosanoid pathway, expression of COX-2 was inhibited by 15% by SCA D and G and up to 90% by SCA E. The IC_{50} for the formation of COX-2-derived PGE_2 was 15 μM (SCA D [Heo et al., 2014]), 12.5 μM (SCA E [Lee et al., 2013]), and 20 μM (SCA G [Yoon et al., 2012a]), respectively. The LPS-induced production of TNF-α, IL-6 and IL-1β was effectively blocked by SCA D (IC_{50} >60, >20–25, and 40 μM, respectively

[Heo et al., 2014]), E (IC_{50} >25 μM, not investigated and >15 μM, respectively [Lee et al., 2013]), and G (IC_{50} 40, 20, and 20 μM, respectively [Yoon et al., 2012a]). The total inflammatory capacity, as determined by the expression of iNOS and COX-2, the production of their respective signaling molecules, nitric oxide and PGE_2, as well as the production of cytokines leads to the following estimation of compound effectiveness: SCA E > D > G.

Amplexichromanols

Amplexichromanols can be distinguished as α-, β-, γ-, δ-forms. δ-Amplexichromanols have been shown to inhibit the secretion of TNF-α (IC_{50} <10 μM) and IL-1β (IC_{50} 10 μM) in LPS-stimulated monocytes (Richomme et al., 2017). To the best of our knowledge, there are no reports on anti-inflammatory effects of the other forms of AC.

Chromenols

Compared to the complex group of structures comprising the chromanol family, chromenol structures are less ubiquitous. Sargachromenol is described here as a representative of the chromenols with anti-inflammatory effects. An ethanolic extract of *Myagropsis myagroides* inhibited nitric oxide-, eicosanoid-, and cytokine-mediated pathways and the inflammatory response (**Table 2**), with sargachromenol being the lead compound in the extract (Kim et al., 2014). Further studies using isolated sargachromenol from different sources confirmed the results obtained by Kim et al. For instance, sargachromanol isolated from the marine brown alga *Sargassum serratifolium* inhibited peroxinitrite anion-mediated albumin nitration with an IC_{50} of 5 μM (Ali et al., 2017). Furthermore, the COX-2 pathway was inhibited using 50 μM and 100 ppm sargachromenol isolated from *Sargassum micracanthum* (Yang et al., 2013) and *Iryanthera juruensis* seeds (Silva et al., 2007), respectively. Here, the effect sizes of 70 and 84% found by Yang *et al.* and Silva *et al.*, respectively, are comparable with respect to the inhibition of the expression of COX-2 protein. For the respective signaling molecule PGE_2 an IC_{50} value of 30 μM was defined (Yang et al., 2013). In addition, inhibitory effects were observed for the expression of iNOS protein (95%) and the formation of nitric oxide (IC_{50} 82 μM) (Yang et al., 2013).

Carcinogenesis

For the evaluation of anti-carcinogenic effects of chromanol and chromenol structures, key apoptotic pathways, such as cleavage of poly-[ADP-ribose]-polymerase 1 (PARP-1), caspases 3, 7, 8, and 9 as well as anti-proliferative and cytotoxic properties on cancer cell lines and further markers of carcinogenesis marker in mice were evaluated (**Figure 7**). In addition, large-scaled human trials investigating preventive and therapeutic effects of some tested compounds will be discussed in the following chapter.

Chromanols

A detailed overview on the biological activities of chromanols linked to carcinogenesis is provided in **Table 3**.

Tocopherols and Tocotrienols

Like the mediation of anti-inflammatory effects, anti-carcinogenic actions were profoundly investigated for α-TOH

TABLE 2 | Overview on the biological activities of chromenols linked to inflammation.

Nitric oxide		Eicosanoid-mediated		Cytokine-mediated		
iNOS	**NO**	**COX-2**	**PGE2**	**IL-1β**	**IL-6**	**TNF-α**
Sargachromenol						
LPS iNOS PE BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS NO PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS COX-2 E BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS PGE_2 PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS IL-1β PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS IL-6 PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)	LPS TNF-α PrD BV-2 cells 2.7 μM inhibition (Kim et al., 2014)
LPS iNOS PE RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS NO PrD RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS COX-2 PE RAW264.7 50 μM inhibition (Yang et al., 2013)	LPS PGE_2 PrD RAW264.7 50 μM inhibition (Yang et al., 2013)			
	peroxynitrite NO PrD BSA nitrition 2.5 μM inhibition (Ali et al., 2017)	– COX-2 A enzyme 100 ppm inhibition (Silva et al., 2007)				

The effects of the respective compounds on inflammation have been divided into activities mediated by nitric oxide (iNOs, NO), eicosanoids (COX-2, PGE_2), and cytokines (IL-1β, IL-6, TNF-α). The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. In the publications where no stimulus was used or was required for the studies, the respective row is marked with a dash. The following abbreviations are used: A, activity; BSA, bovine serum albumin; BV-2, brain microglial cells transformed by recombinant retrovirus (v-raf/v-mic); COX-2, Cyclooxygenase 2; E, expression; iNOS, induce able nitric oxide synthase; IL-1β, interleukin 1β; IL-6, interleukin 6; LPS, lipopolysaccharides; m, murine; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; NO, nitric oxide; PrD, production; PGE_2, prostaglandin E_2; PE, protein expression; TNF-α, tumor necrosis factor α.

TABLE 3 | Overview on the biological activities of chromanols linked to carcinogenesis.

Apoptosis/Necrosis mediated					Proliferation			Viability
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**				
α-TOH								
PARP-1 CL MDA-MB-231 MCF-7 cells 23 µM no induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM no induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 µM no induction (Husain et al., 2011)	Casp7 CL SW 480 cells HCT-116 100 µM no induction (Campbell et al., 2006)	MDA-MB-435 > 2000 µM MCF-7 cells 290 µM no inhibiton (Guthrie et al., 1997)	MDA-MB-435 230 µM no inhibition (Nesaretnam et al., 1995)		h_cc cells 200 µM no reduction (Campbell et al., 2006)
PARP-1 CL SW 480 cells HCT-116 100 µM no induction (Campbell et al., 2006)	Casp8 CL SW 480 cells HCT-116 100 µM no induction (Campbell et al., 2006)		Casp3 CL SW 480 cells HCT-116 100 µM no induction (Campbell et al., 2006)		m_NB2A cells inhibition h_ SaOs-2 cells no inhibition 50 µM (Azzi et al., 1993)	Du-145 cells LNCaP cells CaCo-2 cells 25 µM inhibition SaOs-2 cells no inhibition (Gysin et al., 2002)		MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)
					PC-3 HTB-82 50 µM inhibition (Galli et al., 2004)	MCF-7 cells 23 µM no inhibition (Nesaretnam et al., 1998)		
					MDA-MB-231 MCF-7 cells 46.5 µM no inhibition (Loganathan et al., 2013)	HT-29 100 µM inhibition (Campbell et al., 2006)		
β-TOH								
					Du-145 cells LNCaP cells SaOs-2 cells 25 µM inhibition (Gysin et al., 2002)	m_NB2A cells h_ SaOs-2 cells 50 µM no inhibition (Azzi et al., 1993)		MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)
γ-TOH								
PARP-1 CL SW 480 cells HCT-116 100 µM induction (Campbell et al., 2006)	Casp8 CL SW 480 cells HCT-116 100 µM induction (Campbell et al., 2006)		Casp3 CL SW 480 cells HCT-116 100 µM induction (Campbell et al., 2006)	Casp7 CL SW 480 cells HCT-116 100 µM induction (Campbell et al., 2006)	PC-3 cells HTB-82 cells 1 µM inhibition (Galli et al., 2004)	h_cc cells 100 µM inhibition (Campbell et al., 2006)	HCT-116, HT-29 50 µM no inhibition (Jang et al., 2016)	h_cc cells 100 µM reduction (Campbell et al., 2006)
					Du-145 cells LNCaP cells CaCo-2 cells SaOs-2 cells 25 µM inhibition (Gysin et al., 2002)	tumor count m_BALB/c 0.1% diet reduction (Jiang et al., 2013)	PC-3, LNCaP 50 µM inhibition (Jiang et al., 2012)	MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)
δ-TOH								
							MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)	HCT-116 inhibition HT-29 no reduction 50 µM (Jang et al., 2016)
α-T3								

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation		Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**				
PARP-1 CL, MDA-MB-231, MCF-7 cells 23.5 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		MDA-MB-435 211.9 µM MCF-7 cells 14.1 µM inhibition (Guthrie et al., 1997)	m_B16(F10) 110 µM inhibition (He et al., 1997)	SCID mice 200 mg/kg no reduction (Husain et al., 2011)	MiaPaCa-2, 50 µM no reduction (Husain et al., 2011)
PARP-1 CL MiaPaCa-2 50 µM no induction (Husain et al., 2011)					MDA-MB-231 22.5 µM MCF-7 cells 26.1 µM inhibition (Loganathan et al., 2013)	MCF-7 23.5 µM no inhibition (Nesaretnam et al., 1998)		MCF-7, MCF-7-C3 50 µM no reduction (Birringer et al., 2003)
β-T3								MiaPaCa-2, 50 µM reduction (Husain et al., 2011)
γ-T3								
PARP-1 CL, MDA-MB-231, MCF-7 cells 24.2 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)	Casp9 CL PC-3, LNCaP 20 µM induction (Jiang et al., 2012)	Casp3 A MCF-7, MCF-7-C3 50 µM induction (Birringer et al., 2003)	Casp7 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	SKBR3, BT474 5 µM inhibition (Alawin et al., 2016)	rh_RLh-84 50 µM inhibition (Sakai et al., 2004)	MiaPaCa-2, 50 µM reduction (Husain et al., 2011)	PC-3, LNCaP 20 µM reduction (Jiang et al., 2012)
PARP-1 CL MiaPaCa-2 50 µM induction (Husain et al., 2011)	Casp8 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	Casp9 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)	Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		m_B16(F10) 20 µM inhibition (He et al., 1997)	PC-3 32 µM inhibition (Yap et al., 2008)		
PARP-1 CL PC-3, LNCaP 20 µM induction (Jiang et al., 2012)	Casp8 CL rh_RLh-84 25 µM induction (Sakai et al., 2004)		Casp3 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)		MDA-MB-231 11.4 µM MCF-7 cells 15.4 µM inhibition (Loganathan et al., 2013)	MCF-7 14.6 µM inhibition (Nesaretnam et al., 1998)		
PARP-1 CL PC-3, LNCap 30/90 µM induction (Yap et al., 2008)			Casp3 CL rh_RLh-84 25 µM induction (Sakai et al., 2004)		MDA-MB-435 73.2 µM MCF-7 cells 4.9 µM inhibition (Guthrie et al., 1997)			
δ-T3								
PARP-1 CL, MDA-MB-231, MCF-7 cells 25.2 µM induction (Loganathan et al., 2013)	Casp8 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		Casp3 A MiaPaCa-2 50 µM induction (Husain et al., 2011)		MDA-MB-435 226.8 µM MCF-7 cells 5 µM inhibition (Guthrie et al., 1997)	PC-3 41 µM LNCap 75 µM inhibition (Yap et al., 2008)		MiaPaCa-2, 50 µM reduction (Husain et al., 2011)
PARP-1 CL MiaPaCa-2 50 µM induction					m_B16(F10) 10 µM inhibition (He et al., 1997)	MCF-7 25.2 µM inhibition (Nesaretnam et al., 1998)		

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation	Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**			
(Husain et al., 2011)							
					MDA-MB-231 MCF-7 cells 17 µM inhibition (Loganathan et al., 2013)		
α-T-13'-OH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 µM no induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 µM no induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 µM no induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 µM no induction (Birringer et al., 2010)		m_C6 cells 10 µM reduction (Mazzini et al., 2009)	THP-1 MΦ 100 µM no reduction (Wallert et al., 2014a)
α-T-13'-COOH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 µM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 µM induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		THP-1 MΦ 7.4 µM reduction (Wallert et al., 2014a)	HepG2 cells 13.5 µM reduction (Birringer et al., 2010)
δ-T-13'-OH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 µM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 µM no induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		THP-1 100 µM no reduction (Schmölz et al., 2017)	HepG2 cells 50 µM no reduction (Birringer et al., 2010)
δ-T-13'-COOH (tocopherol derived)							
PARP-1 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		Casp9 CL HepG2 cells 20 µM induction (Birringer et al., 2010)	Casp3 CL HepG2 cells 20 µM induction (Birringer et al., 2010)	Casp7 CL HepG2 cells 20 µM induction (Birringer et al., 2010)		HCT-116 HT-29 8.9/8.6 µM reduction (Jang et al., 2016)	HepG2 cells 6.5 µM reduction (Birringer et al., 2010)
PARP-1 CL HCT-116 20 µM induction (Jang et al., 2016)		Casp9 CL HCT-116 20 µM induction (Jang et al., 2016)				m_C6 cells 10 µM reduction (Mazzini et al., 2009)	HCT-116 HT-29 8.9/8.6 µM reduction (Jang et al., 2016)
						THP-1 11.1 µM reduction (Schmölz et al., 2017)	
α-T-3'-COOH (tocopherol derived)							
					PC-3 cells HTB-82 cells 1 µM inhibition (Galli et al., 2004)		m_C6 cells 10 µM reduction (Mazzini et al., 2009)
γ-T-3'-COOH (tocopherol derived)							
					PC-3 cells HTB-82 cells 1 µM inhibition (Galli et al., 2004)		m_C6 cells 10 µM reduction (Mazzini et al., 2009)
δ-T3-13′-COOH							

(Continued)

TABLE 3 | Continued

Apoptosis/Necrosis mediated					Proliferation	Viability	
PARP-1	**Casp8**	**Casp9**	**Casp3**	**Casp7**			
PARP-1 CL HCT-116 20 µM induction (Jang et al., 2016)		Casp9 CL HCT-116 20 µM induction (Jang et al., 2016)				m_C6 cells 10 µM reduction (Mazzini et al., 2009)	HCT-116 HT-29 16/17 µM reduction (Jang et al., 2016)
SCA E							
PARP-1 CL h_HL-60 25 µM induction (Heo et al., 2011)		Casp9 CL h_HL-60 25 µM induction (Heo et al., 2011)	Casp3 CL h_HL-60 25 µM induction (Heo et al., 2011)		h_HL-60 50 µM inhibition (Heo et al., 2011)		
α-AC							
							HepaRG 10 µM no reduction (Richomme et al., 2017)

The effects of the respective compounds on carcinogenesis have been divided into apoptosis-mediated (PARP-1, caspases 3, 7, 8, and 9) activities as well as activities affecting proliferation and viability. The content of each cell of the table in the apoptosis section is constructed as follows (read from top to bottom): (i) investigated parameter; (ii) cell type model used for investigation; (iii) used concentration of the respective compound; (iv) observed effect on the studied parameter; (v) reference. The content of each table cell in the proliferation as well as viability section is constructed as follows (read from top to bottom): (i) cell type model used for investigation; (ii) used concentration of the respective compound; (iii) observed effect on the studied parameter; (iv) reference. The following abbreviations are used: A, activity; A549, adenocarcinomic human alveolar basal epithelial cells; BALB/c mice, albino laboratory-bred strain of the house mouse; LNCap, androgen-sensitive human prostate adenocarcinoma cells; Casp, caspase; MCF-7-C3, caspase 3 reconstituted MCF-7 cells; CL, cleavage; cc, colon cancer; MDA-MB-23, epithelial human breast cancer cell line; NB2A, fast-growing mouse neuroblastoma cell line; h, human; MCF-7, human breast cancer cell line established by Michigan Cancer Foundation-7; SKBR3, human breast cancer cell line isolated by the Memorial Sloan–Kettering Cancer Centere; BT-474, human breast carcinoma ductal cell line; SaOs-2, human cell line derived from primary osteosarcoma; SW-480, human cell line established from a lymph node metastasis; HCT-116, human colon cancer cell line; HT-29, human colorectal adenocarcinoma cell line; CaCo-2, human epithelial colorectal adenocarcinoma cells; THP-1, human immortalized monocyte-like cell line; HL-60, human leukemia cell line; HepG2, human liver cancer cell line; MiaPaCa-2, human pancreatic cancer cell line; Du-145, human prostate cancer cell line; PC-3, human prostate cancer cell line; B16(10), mouse skin melanoma cells; m, murine; m_C6, murine glial cancer cell line; PARP-1, poly (ADP-Ribose)-polymerase 1; HTB-82, rhabdomyosarcoma cell line; rh_RLh-84, rat hepatoma cell line; HepaRG, terminally differentiated hepatic cells derived from a human hepatic progenitor cell line.
All results obtained from in vivo studies are marked in gray.

and γ-TOH, due to their biological relevance. Screening of multiple human breast cancer cell lines (Nesaretnam et al., 1995; Guthrie et al., 1997; Nesaretnam et al., 1998; Birringer et al., 2003; Loganathan et al., 2013) and the human osteosarcoma cell line Saos-2 (Azzi et al., 1993; Gysin et al., 2002) revealed no anti-proliferative effects or alteration of cell viability using α-TOH (4.6–230 µM), whereas Campbell et al. found controversial results for different human colon cancer cell lines using different assays (Campbell et al., 2006). However, the colon cancer cell lines HT-29 (Campbell et al., 2006) and CaCo-2 (Gysin et al., 2002) treated with 100 µM (48 h), 200 µM (5 h), and 25 µM (24 h) α-TOH showed significantly induced cell death and dampened proliferation. In addition, proliferation of different human prostate cancer cells was significantly inhibited by α-TOH. In brief, 25–50 µM α-TOH inhibited proliferation of PC-3 cells (41%, 24 h) (Galli et al., 2004), Du-145 cells (50%, 24 h), and LNCaP cells (48%, 48 h) (Gysin et al., 2002). Furthermore, α-TOH (50 µM) significantly inhibited the growth of murine neuroblastoma NB2A cells by 50% (Azzi et al., 1993), and rhabdomyosarcoma HTB-82 cells by 32% (Galli et al., 2004). However, effects on proliferation and viability seemed to be independent from the cleavage and activity of the apoptosis marker PARP-1 and caspases 3, 7, and 8 in breast cancer (23 µM [Loganathan et al., 2013]), pancreas cancer (50 µM [Husain et al., 2011]), and colon cancer cells (100 µM [Campbell et al., 2006]). β-TOH-treatment of cancer cells revealed similar effects compared to α-TOH. While growth of human prostate cancer cells was significantly inhibited by >40% (Gysin et al., 2002), growth of human osteosarcoma cells was marginally inhibited. In neuroblastoma (Azzi et al., 1993) and breast cancer cells (Birringer et al., 2003) β-TOH did not alter cell viability.

γ-Tocopherol is by far the most potent anti-carcinogenic TOH regarding prostate-cancer. Indeed, viability or rather proliferation of prostate-cancer cell lines PC-3 (1 µM [Galli et al., 2004], 50 µM [Jiang et al., 2012]), CaCo-2, Du-145, LNCaP (25 µM [Gysin et al., 2002]), SW480, HCT-116, HCT-15, and HCT-29 (100 µM [Campbell et al., 2006]) was blocked by γ-TOH. More precisely, 100 µM γ-TOH induced apoptosis in SW480 and HCT-116 cells following the cleavage of PARP-1 as well as caspases 3, 7, and 8 (Campbell et al., 2006). Described effects are most likely tumor-specific, finding no or weak alteration of tumor growth on breast cancer cell lines (Birringer et al., 2003) and colon carcinoma cells (Jang et al., 2016). However, in male BALB/c mice γ-TOH (0.1% of diet) suppressed DSS- and AOM-induced tumor multiplicity of macroscopic adenomas and large adenomatous polyps (>2mm^2) by 60, 85, and 36% (Jiang et al., 2013). Of the tested tumor cell lines, only viability of HCT-116 was inhibited by 50

μM δ-TOH, whereas HT-29 cells, and the breast cancer cell lines MCF-7 and MCF-7-C3 were not affected (Birringer et al., 2003; Jang et al., 2016). Based on the presented data, anti-carcinogenic capacity for different forms of TOHs can be assessed as γ-TOH >> β-TOH > α/δ-TOH.

Despite of the promising results outlined above, it should be noticed that several human trials failed to confirm preventive effects of vitamin E, in particular α-TOH, against cancer. The Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention Study examined whether a daily supplementation of 50 mg α-TOH and/or 20 mg β-carotene could prevent lung cancer in male smokers (Virtamo et al., 2014). However, after five to eight years of supplementation of either α-TOH or β-carotene or the combination of both failed to prevent lung cancer (Virtamo et al., 2014). In addition, other human intervention trails revealed disappointing results, with the Selenium and Vitamin E Cancer Prevention Trial (SELECT) representing a very interesting one. The aim of the SELECT study was to investigate the preventive potential of α-TOH and/or selenium on prostate cancer. In the SELECT trial, healthy men received a daily dose of either 400 IU all-*rac*-α-tocopheryl acetate or 200 μg selenium or a combination of both for an average of 5.5 years (Lippman et al., 2009). Supplementation with both compounds failed to prevent prostate cancer development. Surprisingly, daily supplementation with all-*rac*-α-tocopheryl acetate was slightly, but not significantly, associated with an increased overall risk for prostate cancer (Lippman et al., 2009). Next, in the 7 to 12 years follow-up the subjects who had received a daily dose of 400 IU all-*rac*-α-tocopheryl acetate showed a significantly enhanced risk for prostate cancer (Klein et al., 2011). This result indicates that a dietary supplementation with high doses of this vitamin E derivate could result in an increased risk for cancer.

The T3-rich fraction of palm oil is comprised of all T3 forms (α- [25%], γ- [29%], δ-T3 [14%] relative to the total vitamin E amount) and inhibits the proliferation of the estrogen receptor-negative human breast cancer cell line MDA-MB-435 with an IC_{50} of 180 μg/ml (Nesaretnam et al., 1995). Based on that finding, single forms of T3s were tested regarding their effects on proliferation and viability of carcinoma cell lines. The α-, γ-, and δ-forms of T3s were found to mediate cancer type specific effectiveness, with breast cancer cell lines being most affected by the treatment with TOHs. Viability and proliferation of MDA-MB-231 (IC_{50} 22.5 μM), MCF-7 (IC_{50} 14.1–26.1 μM), and MDA-MB-435 cells (IC_{50}: 211.9 μM) were concentration-dependently affected by α-T3 treatment independent on whether they were responsive to estrogen and estradiol (Guthrie et al., 1997; Nesaretnam et al., 1998; Loganathan et al., 2013). However, whereas cleavage of PARP-1 (Loganathan et al., 2013) has been observed, general involvement of apoptosis has not been described yet (Birringer et al., 2003). Although cleavage of PARP-1 as well as caspases 3 and 8 has been observed in pancreatic MiaPaCa-2 carcinoma cells, 50 μM α-T3 had no effect on cell viability (Husain et al., 2011). In contrast, β-T3 (50 μM) reduced the viability of MiaPaCa-2 cells (Husain et al., 2011). In mice, 200 mg/kg α-T3 did not affect tumor growth of AsPC-1 human pancreatic cancer xenografts (Husain et al., 2011), whereas 110 μM α-T3 suppressed proliferation of murine B16(F10) melanoma cells (He et al., 1997).

Within the group of TOHs and T3s, γ-T3 is the most potent anti-carcinogenic form that affects cell growth of breast, prostate, pancreas, and hepatic cancer cells, likely due to a preferred incorporation of γ-T3 in these cells (Sakai et al., 2004). There is strong evidence for the anti-proliferative effects of γ-T3 on breast cancer cell lines MDA-MB-231 (IC_{50} 11.4 μM), MCF-7 (IC_{50} 15.4 μM) (Loganathan et al., 2013), SKBR3 (IC_{50} 4 μM), BT474 (IC_{50} 4 μM) (Alawin et al., 2016), estrogen receptor-negative MDA-MB (IC_{50} 73.2 μM), and estrogen receptor-positive MCF-7 cells (IC_{50} 4.9 μM) (Guthrie et al., 1997). Others even found complete inhibition of MCF-7 cell growth by γ-T3 at a concentration of 14.6 μM (Nesaretnam et al., 1998). Inhibitory effects on proliferation were at least in part mediated *via* the activation of apoptosis, such as activation of caspase 3 in MCF-7 (25%), and MCF-7-C3 cells (35%) with 50 μM γ-T3 (Birringer et al., 2003). Furthermore, the proliferation of MiaPaCa-2 pancreas cancer cells (Husain et al., 2011), PC-3 prostate cancer cells (IC_{50}: 32 μM, 24 h), and dRLh-84 hepatic cancer cells (IC_{50}: 80–100 μM, 24 h) was suppressed by γ-T3, most likely *via* cleavage of PARP-1, and caspases 3, 7, 8, and 9 (Sakai et al., 2004; Yap et al., 2008) and induction of autophagy (Jiang et al., 2012). In murine B16(F10) melanoma cells (He et al., 1997) and the myelogenous leukemia cell line KBM-5 (Ahn et al., 2007) γ-T3 significantly suppressed proliferation (IC_{50} 20 μM, 24 h). Comparable to γ-T3, δ-T3 inhibits the proliferation of the breast cancer cell lines MDA-MB-435 (IC_{50} 226.8 μM), MDA-MB-231 (IC_{50} 17.4 μM), and MCF-7 cells (IC_{50} 5–25.2 μM) (Guthrie et al., 1997; Nesaretnam et al., 1998; Loganathan et al., 2013), as well as prostate cancer cell lines PC-3 (IC_{50} 41 μM), and LNCaP (IC_{50} 75 μM) (Yap et al., 2008), melanoma B16(F10) cells (IC_{50} 10 μM) (He et al., 1997), and MiaPaCa-2 pancreas cancer cells (IC_{50} 50 μM) (Husain et al., 2011) by the induction of apoptosis, as indicated by the cleavage of apoptosis-mediating PARP-1 as well as caspases 3 and 8 (Husain et al., 2011).

Metabolites of Tocopherols and Tocotrienols

In contrast to the TOH and T3 forms, the respective metabolites have been rarely investigated regarding their anti-carcinogenic properties. The LCMs of TOHs, namely α-T-13′-COOH (20 μM) and δ-T-13′-COOH (20 μM) induced apoptosis *via* the mitochondrial pathway, which was shown by cleavage of PARP-1 and caspases 3, 7, and 9, resulting in decreased viability of HepG2 cells (IC_{50} 13.5 μM and 6.5 μM, respectively, Birringer et al., 2010). In human leukemia-derived THP-1 macrophages, viability was decreased by α-T-13′-COOH (IC_{50} 7.4 μM, Wallert et al., 2014a) and δ-T-13′-COOH (IC_{50} 11.1 μM, Schmölz et al., 2017). In addition, δ-T-13′-COOH increased apoptosis-induced cytotoxicity in HCT-116 (IC_{50} 8.9 μM), HT-29 (IC_{50} 8.6 μM) (Jang et al., 2016), and C6 cells (IC_{50} <10 μM, Mazzini et al., 2009). The T3-derived δ-garcinoic acid decreased the viability of HCT-116, HT-29 (Jang et al., 2016), glioma C6 (Mazzini et al., 2009), and human THP-1 macrophage-like cells (IC_{50} <20 μM, unpublished data) to a similar extent. In BALB/c mice fed with 0.022%, δ-garcinoic acid in the diet, AOM- and DSS-induced

colon tumor growth was decreased (Jang et al., 2016). In contrast to the carboxychromanol structures, the hydroxychromanols were less efficient in the cleavage of apoptosis markers and consequently did not affect the viability of HepG2 cells (Birringer et al., 2010) and THP-1 macrophages (Wallert et al., 2014a; Schmölz et al., 2017) at concentrations up to 50 μM and 100 μM, respectively, whereas an anti-proliferative effect on glioma C6 cancer cells was determined using 10 μM α-T-13′-OH (Mazzini et al., 2009). Short-chain metabolites were found to affect growth of prostate cancer cells PC-3 and rhabdomyosarcoma HTB-82 cells at a concentration of 1 μM (Galli et al., 2004).

Sargachromanols

The group of sargachromanols may serve as anti-carcinogenic agents that suppress cell proliferation as reported for SCA E in HL-60 leukemia cells accompanied by cleavage of PARP-1 as well as caspases 3 and 9 (Heo et al., 2011). However, confirmatory data are pending.

Amplexichromanols

To date, α-AC has been studied only in HepaRG cells, without effects on viability up to concentrations of 10 μM (Richomme et al., 2017). Therefore, studies on anti-carcinogenic effects of amplexichromanols are still on demand.

Chromenols

Within the group of chromenols, δ-sargachromenol is the best-studied one. Previous studies revealed an induction of the cleavage of PARP-1 and caspases along with the induction of apoptosis and reduced cell viability in human skin keratinocyte (HaCaT) cells (Hur et al., 2008). Data obtained from cancer cell lines is still lacking.

INTERFERENCE WITH MOLECULAR TARGETS AND KEY PROTEINS CONNECTING INFLAMMATION AND CARCINOGENESIS

Many signaling molecules involved in inflammatory processes play in parallel also key roles in carcinogenesis. We here exemplarily focus on the interaction of selected chromanols and chromenols with the molecular crosstalk of NF-κB (Jurjus et al., 2016), lipoxygenases (Rådmark et al., 2015; Roos et al., 2016; Merchant et al., 2018), MAPK (Gkouveris and Nikitakis, 2017; Jiménez-Martínez et al., 2019), and the inflammasome (Moossavi et al., 2018; Swanson et al., 2019) due to their accepted involvement in both, inflammation and cancer (**Figure 7**). However, due to the sparse knowledge about their connection to chromanols and chromenols, further topics, like the interaction of tumor and immune cells, adhesion proteins, structure and regulation of tumor microenvironments, mechanisms for programed cell death as well as other prominent signaling pathways (PI3K/Akt/mTOR; PKC; STAT; Wnt/β-catenin), were not considered in this review.

Chromanols

A detailed overview on the interference of chromanols with molecular targets and key enzymes connecting inflammation and carcinogenesis is provided in **Table 4**.

Tocopherols and Tocotrienols

As outlined above, inflammation and carcinogenesis are only marginally affected by α-TOH. This is probably the consequence of a lack of interference of α-TOH with NF-κB. Neither in phorbol-12-myristat-13-acetate (PMA)-stimulated BALBc/3T3 fibroblasts (Azzi et al., 1993), and human pancreatic cancer MiaPaCa-2 cells (Husain et al., 2011), nor TNF-α-stimulated murine myelogenous leukemia KBM-5 cells (Ahn et al., 2007), α-TOH (50 μM), β-TOH (50 μM), or γ-TOH (25 μM) affected NF-κB binding affinity or its activation. In murine RAW264.7 macrophages, 100 μM α-TOH even induced translocation of p65 into the nucleus (Wallert et al., 2015). However, pharmacological doses of α-TOH (500 μM) inhibited NF-κB transcriptional activity as well as the phosphorylation and subsequent degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB)-α, the inhibitor of NF-κB, resulting in decreased NF-κB activation in multifactorially stimulated dendritic cells (Tan et al., 2005). γ-Tocotrienol and δ-T3 significantly decreased NF-κB/p65 binding affinity in MiaPaCa-2 cells and diminished p65 subunit translocation in AsPc-1 cells and tumor tissue. In addition, β-T3 and δ-T3 inhibited the translocation in MiaPaCa-2 cells (Husain et al., 2011). The NF-κB inhibitor IκB-α remained unchanged in the aforementioned study. Within the group of T3s, γ-T3 has been described to affect NF-κB activation and p65 subunit translocation in various cell lines and isolated tissue. For example, γ-T3 (20–40 μM) inhibited the phosphorylation of IκB-α and the nuclear translocation of the p65 subunit following various stimuli, including pro-inflammatory cytokines, tumor promoters, carcinogens, and growth factors in different cell lines (Ahn et al., 2007; Yap et al., 2008; Wang et al., 2015). Further, γ-T3 treatment also increased IκB-α protein expression in epididymal adipose tissues isolated from γ-T3-fed *db/db* mice (Kim et al., 2016) as well as in LPS/palmitate-activated BMDM using 1 μM γ-T3 (Kim et al., 2018). In mice, 400 mg γ-T3/kg, applied orally, sensitized pancreatic tumors to gemcitabine treatment, a drug applied in clinical treatment of pancreatic cancer, by suppressing NF-κB-mediated inflammatory pathways linked to tumorigenesis (Kunnumakkara et al., 2010). The expression of A20 (acronym: TNFAIP3), another inhibitor of NF-κB, was induced by 20 μM γ-T3 in RAW264.7, A549, PC3, and MCF-7 cells (Wang et al., 2015) as well as in peritoneal macrophages obtained from diabetic *db/db* mice fed with a γ-T3-containing diet (0.1%) (Kim et al., 2016).

5-, 12-, and 15-LO pathways mediate the formation of lipid mediators (including leukotrienes, lipoxins, resolvins, protectins, and maresins), which orchestrate inflammation by triggering immune cell recruitment and allergic responses, and/or actively terminating inflammation, i.e. triggering resolution of inflammation. Leukotrienes and the so-called specialized pro-resolving lipid mediators (produced by the tumor-

TABLE 4 | Overview on the interference of chromanols with molecular targets and key enzymes connecting inflammation and carcinogenesis.

NF-κB				NLRP3	MAPKs	Lipoxygenases	
α-TOH							
PMA NF-κB A BALB c/3T3 fibroblasts 50 μM no inhibition (Azzi et al., 1993)	LPS NF-κB PE (Nucleus) RAW264.7 100 μM induction (Wallert et al., 2015)	IL-1β, IL-6, TNF-α, LPS, PGE_2, INF-γ h_DC NF-κB A 500 μM inhibition (Tan et al., 2005)	IL-1β, IL-6, TNF-α, LPS, PGE_2, INF-γ h_DC IκB-α Phos 500 μM inhibition (Tan et al., 2005)		– 5-LO A enzyme 5 μM inhibition (Reddanna et al., 1985)	– 5-LO A enzyme >1 μM inhibition (Pein et al., 2018)	AA 5-LO PF PMNL 808 μM inhibition (Pein et al., 2018)
– p65 DNA BA MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)					AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	
β-TOH							
PMA NF-κB A Balb c/3T3 fibroblasts 50 μM no inhibition (Azzi et al., 1993)					– 5-LO A enzyme 750 nM inhibition (Pein et al., 2018) AA 5-LO PF PMNL 57 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
γ-TOH							
TNF-α NF-κB Actv KBM-5 25 μM no inhibition (Ahn et al., 2007)					– 5-LO A enzyme 2–3 μM inhibition (Reddanna et al., 1985) – 5-LO A enzyme > 50 μM no inhibition (Jang et al., 2016) – 5-LO A enzyme 200 μM inhibition (Jiang et al., 2011)	AA 5-LO PF PMNL 502 μM inhibition (Pein et al., 2018) AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	– 5-LO A enzyme 910 nM inhibition (Pein et al., 2018) AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
δ-TOH							
					– 5-LO A enzyme 310 nM inhibition (Pein et al., 2018)	– 5-LO A enzyme > 50 μM no inhibition (Jang et al., 2016)	

(Continued)

TABLE 4 | Continued

NF-κB				NLRP3	MAPKs	Lipoxygenases		
						AA 5-LO PF PMNL 85 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM inhibition (Pein et al., 2018)
α-T3								
– p65 DNA BA MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl AsPC-1 50 μM no inhibition (Husain et al., 2011)	– p65 Trl m_TT 50 μM no inhibition (Husain et al., 2011)			– 5-LO A enzyme 330 nM inhibition (Pein et al., 2018) AA 5-LO PF PMNL 277 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
β-T3								
– p65 DNA BA (Cytosol) MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 DNA BA (Nucleus) MiaPaCa-2 50 μM no inhibition (Husain et al., 2011)	– p65 Trl MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 Trl m_TT 50 μM no inhibition (Husain et al., 2011)			– 5-LO A enzyme 190 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
– p65 Trl AsPC-1 50 μM no inhibition (Husain et al., 2011)						AA 5-LO PF PMNL 95 μM inhibition (Pein et al., 2018)		
γ-T3								
– p65 Trl PC3 cells 40 μM inhibition (Yap et al., 2008)	TNF-α NF-κB Actv RAW264.7 20 μM inhibition (Wang et al., 2015)	TNF-α NF-κB Actv H1299, A293, MCF-7, U226, SCC4 25 μM inhibition (Ahn et al., 2007)	diabetes IκB-α PE *db/db* mice 0.1% of diet induction (Kim et al., 2016)	LPS/pal, Ng NLRP3 E m_BMDM 1 μM inhibition (Kim et al., 2016)	diabetes p38 Phos db/db mice 0.1% of diet inhibition (Kim et al., 2016)	- 5-LO A enzyme 200 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
– NF-κB Actv mice 400 mg/kg/d inhibition (Kunnumakkara et al., 2010)	– p65 DNA BA MiaPaCa-2 50 μM inhibition (Husain et al., 2011)	– p65 Trl AsPC-1 50 μM inhibition (Husain et al., 2011)	TNF-α IκB-α PE RAW264.7, m_BMDM 20 μM induction (Wang et al., 2015)	diabetes NLRP3 E db/db mice derived PM/AT 0.1% of diet inhibition (Kim et al., 2016)	diabetes ERK Phos db/db mice 0.1% of diet inhibition (Kim et al., 2016)	AA 5-LO PF PMNL 132 μM inhibition (Pein et al., 2018)	LPS/pal 5-LO GE BMDM 1 μM no inhibition (Kim et al., 2018)	
– p65 Trl MiaPaCa-2 50 μM no inhibition	– p65 Trl m_TT 50 μM inhibition	TNF-α IκB-α Phos A549, PC3, MCF-7 20 μM inhibition (Wang et al., 2015)	LPS/pal IκB-α PE m_BMDM 1 μM induction (Kim et al., 2018)		TNF-α JNK Phos RAW264.7, m_BMDM 20 μM inhibition			

(Continued)

TABLE 4 | Continued

NF-κB				NLRP3	MAPKs	Lipoxygenases		
(Husain et al., 2011)	(Husain et al., 2011)				(Wang et al., 2015)			
–	–	–			LPS			
IκB-α Phos	IκB-α Phos	IκB-α Phos			ERK Phos			
AsPC-1	MiaPaCa-2	m_TT			m_BMDM			
50 μM	50 μM	50 μM			0.5 μM			
inhibition	inhibition	inhibition			inhibition			
(Husain et al., 2011)	(Husain et al., 2011)	(Husain et al., 2011)			(Kim et al., 2016)			
δ-T3								
–	–	–	–			–	AA	AA
p65 DNA BA	p65 Trl	p65 Trl	IκB-α Phos			5-LO A	12-LO PF	15-LO PF
MiaPaCa-2	AsPC-1	MiaPaCa-1	MiaPaCa-2,			enzyme	PMNL	PMNL
50 μM	50 μM	50 μM	50 μM			170 nM	3 μM	3 μM
inhibition	inhibition	inhibition	inhibition			inhibition	inhibition	no inhibition
(Husain et al., 2011)	(Husain et al., 2011)	(Husain et al., 2011)	(Husain et al., 2011)			(Pein et al., 2018)	(Pein et al., 2018)	(Pein et al., 2018)
–	–	–				AA		
p65 Trl	IκB-α Phos	IκB-α Phos				5-LO PF		
m_TT	MiaPaCa-2,	m_TT				PMNL		
50 μM	50 μM	50 μM				60 μM		
inhibition	inhibition	inhibition				inhibition		
(Husain et al., 2011)	(Husain et al., 2011)	(Husain et al., 2011)				(Pein et al., 2018)		
α-T-13′-OH								
						–	AA	AA
						5-LO A	12-LO PF	15-LO PF
						enzyme	PMNL	PMNL
						350 nM	3 μM	3 μM
						inhibition	inhibition	induction
						(Pein et al., 2018)	(Pein et al., 2018)	(Pein et al., 2018)
						AA		
						5-LO PF		
						PMNL		
						190 nM		
						inhibition		
						(Pein et al., 2018)		
α-T-13′-COOH								
LPS						–	AA	AA
p65 Trl						5-LO A	12-LO PF	15-LO PF
RAW264.7						enzyme	PMNL	PMNL
2.5 μM						270 nM	3 μM	3 μM
no inhibition						inhibition	inhibition	inhibition
(Wallert et al., 2015)						(Pein et al., 2018)	(Pein et al., 2018)	(Pein et al., 2018)
						AA		
						5-LO PF		
						PMNL		
						80 nM		
						inhibition		
						(Pein et al., 2018)		
δ-T-13′-OH								
						–	AA	AA
						5-LO A	12-LO PF	15-LO PF
						enzyme	PMNL	PMNL
						120 nM	3 μM	3 μM
						inhibition	inhibition	induction
						(Pein et al., 2018)	(Pein et al., 2018)	(Pein et al., 2018)

(Continued)

TABLE 4 | Continued

NF-κB	NLRP3	MAPKs	Lipoxygenases	
		AA 5-LO PF PMNL 540 nM inhibition (Pein et al., 2018)		
δ-T-13′-COOH				
		– 5-LO A enzyme >1 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM induction (Pein et al., 2018)
		AA 5-LO PF PMNL 2 μM inhibition (Pein et al., 2018)	– 5-LO A enzyme 2 μM inhibition (Jang et al., 2016)	Ca^{2+} 5-LO PF HL-60 50 μM inhibition (Jiang et al., 2011)
		– 5-LO A enzyme 0.5–1 μM inhibition (Jiang et al., 2011)		
α-T-5′-COOH				
		– 5-LO A enzyme 750 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
α-T-3′-COOH				
		– 5-LO A enzyme >3 μM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL 3 μM inhibition (Pein et al., 2018)	AA 15-LO PF PMNL 3 μM no inhibition (Pein et al., 2018)
δ-T3-13′-COOH				
		– 5-LO A enzyme 35 nM inhibition (Pein et al., 2018)	AA 12-LO PF PMNL > 3 μM no inhibition (Pein et al., 2018)	AA 15-LO PF PMNL > 3 μM no inhibition (Pein et al., 2018)
		AA 5-LO PF PMNL 260 nM inhibition (Pein et al., 2018)	– 5-LO A enzyme 57 nM inhibition (Richomme et al., 2017)	AA 5-LO PF PMNL 345 nM inhibition (Richomme et al., 2017)
		– 5-LO A		

(Continued)

TABLE 4 | Continued

NF-κB			NLRP3	MAPKs	Lipoxygenases
					enzyme 1 μM inhibition (Jang et al., 2016)
SCA D					
LPS p65 Phos RAW264.7 60 μM inhibition (Heo et al., 2014)	LPS IκB-α Phos RAW264.7 60 μM inhibition (Heo et al., 2014)			LPS JNK Phos RAW264.7 30 μM inhibition (Heo et al., 2014) LPS ERK Phos RAW264.7 30 μM inhibition (Heo et al., 2014)	
SCA E					
				LPS ERK Phos RAW264.7 58 μM inhibition (Lee et al., 2013) LPS p38 Phos RAW264.7 58 μM inhibition (Lee et al., 2013) LPS JNK Phos RAW264.7 58 μM inhibition (Lee et al., 2013)	
SCA G					
IL-1β p65/p50 Phos MG-63 40 μM inhibition (Yoon et al., 2012b)		IL-1β IκB-α Phos MG-63 20 μM inhibition (Yoon et al., 2012b)		IL-1β ERK Phos MG-63 40 μM inhibition (Yoon et al., 2012b) IL-1β p38 Phos MG-63 20 μM inhibition (Yoon et al., 2012b) IL-1β JNK Phos MG-63	

(Continued)

TABLE 4 | Continued

NF-κB	NLRP3	MAPKs	Lipoxygenases
		40 μM inhibition (Yoon et al., 2012b)	

The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type, tissue, mouse, or other models used for investigation; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. In the publications where no stimulus was used or was required for the studies, the respective row is marked with "-". Actv, activation; A, activity; AT, adipose tissue; BALB/c mice, albino laboratory-bred strain of the house mouse; AA, arachidonic acid; BA, binding affinity; BMDM, bone marrow derived macrophages; JNK, c-Jun N-terminal kinase; DC, dendritic cells; E, expression; ERK, extracellular-signal regulated kinase; 3T3, murine fibroblast cell line; GE, gene expression; h, human; MCF-7, human breast cancer cell line established by Michigan Cancer Foundation-7; SCC4, human head and neck squamous cell carcinoma cell line; HL-60, human leukemia cell line; H1299, human non-small cell lung carcinoma cell line; MiaPaCa-2, human pancreatic cancer cell line; AsPC-1, human pancreas adenocarcinoma cell line; U226, human peripheral blood myeloma, plasmacytoma cell line; MG-63, human osteosarcoma cell line; INF-γ interferon γ; IL, interleukin; db/db mice, leptin receptor activity deficient mice; LPS, lipopolysaccharide; LO, lipoxygenase; RAW264.7, macrophages derived from abelson murine leukemia virus-induced tumor; MAPK, mitogen-activated protein kinase; m, murine; KBM-5, murine myelogenous leukemia cell line; Ng, nigericine; NLRP3, NLR family pyrin domain containing 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p65, nuclear factor NF-κB p65 subunit; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; pal, palmitate; PM, peritoneal macrophages; PMA, phorbol-12-myristat-13-acetat; Phos, phosphorylation; PMNL, polymorphonuclear neutrophils; PF, product formation; PGE_2, prostaglandin E_2; PE, protein expression; Trl, translocation; TNF-α, tumor necrosis factor α; TT, tumor tissue.

All results obtained from in vivo studies are marked in gray.

microenvironment, in particular by 15-LO-expressing macrophages of the M2 subtype) have further been shown to play pivotal roles in tumor initiation and development as well as angiogenesis and metastasis (Serhan, 2014; Rådmark et al., 2015; Wculek and Malanchi, 2015; Gilligan et al., 2019). All forms of TOHs inhibit the activity of the isolated 5-LO enzyme in the following sequence of their inhibitory capacity: δ-TOH (IC_{50} 0.31 μM) < β-TOH (IC_{50} 0.75 μM) < α-TOH (IC_{50} 1–5 μM) = γ-TOH (IC_{50} 0.9–3 μM) (Reddanna et al., 1985; Pein et al., 2018). In activated polymorphonuclear leukocytes (PMNL), inhibitory concentrations are 10- to100-fold higher with the following order: β-TOH < δ-TOH < γ-TOH < α-TOH (Pein et al., 2018). However, activity of 12- and 15-LO, which catalyze the formation of 12- and 15-HETE, respectively, remained unaltered by 3 μM TOH in LPS-activated PMNL, except for δ-TOH which inhibited 15-LO with an IC_{50} of 3 μM (Pein et al., 2018). α-, β-, γ-, and δ-T3 appeared as efficient inhibitors of isolated 5-LO, all with IC_{50} values below 0.5 μM, whereas the inhibition of 5-LO product formation in activated PMNL required concentrations of 60 μM (δ-T3) to 277 μM (α-T3) (Pein et al., 2018). 12-Lipoxygenase product formation in PMNL was significantly inhibited by all T3 forms, whereas 15-LO-derived products remained unchanged or were even significantly elevated using concentrations of 3 μM (Pein et al., 2018).

MAPK pathways mediate a multitude of cellular processes, including growth, proliferation, differentiation, migration, apoptosis, and inflammation, in response to external stress signals. Therefore, MAPK pathways represent interesting targets for the development of anti-carcinogenic as well as anti-inflammatory therapeutics. Within the MAPK protein family, extracellular signal-regulated kinase (ERK) represents a prominent target for cancer research, because ERK deregulation is linked to approximately one-third of all human cancers (Dhillon et al., 2007). In addition, ERK affects cellular inflammation *via* modulation of cytokine expression (Kim, 2014). However, the stress-activated kinases, c-Jun N-terminal kinase (JNK) and p38, have emerged as interesting therapeutic targets, due to their involvement in the regulation of inflammation, DNA damage response, and apoptosis (Kaminska, 2005). Inhibitory effects of γ-T3 on the MAPK pathway, more precisely the phosphorylation of ERK, p38 and JNK have been observed in epididymal adipose tissues from γ-T3-fed *db/db* mice (0.1% of the diet), in LPS-activated BMDMs using 0.5 μM γ-T3 (Kim et al., 2016), and in TNF-α-activated RAW264.7 cells (Wang et al., 2015). The relevance of NLRP3 inflammasome activation and subsequent formation of pro-inflammatory cytokines, namely IL-1β and IL-8, in inflammation and related diseases has been shown. γ-T3 decreased NLRP3 inflammasome activation by inhibiting the mRNA and protein expression of the NLRP3 inflammasome in BMDM activated with LPS/palmitate, rather than with LPS/nigericin, in peritoneal macrophages and adipose tissue isolated from γ-T3-fed *db/db* mice (Kim et al., 2016). In addition, in BMDMs treated with chloroquine, an inhibitor of lysosomal degradation, the accumulation of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II, and the degradation of p62 were decreased implying that γ-T3 co-regulates autophagosome formation and inflammasome activation (Kim et al., 2016).

Metabolites of Tocopherols and Tocotrienols

Tocopherols and T3s inhibit the activity of isolated recombinant human 5-LO enzyme 10- to 100-fold more efficiently than in activated PMNL. The respective long-chain TOH- and T3-derived metabolites inhibited isolated 5-LO to a similar extent (Jiang et al., 2011; Jang et al., 2016; Pein et al., 2018). Notably, in activated PMNL, α-T-13′-COOH was the most potent inhibitor of 5-LO activity with an IC_{50} value of 80 nM followed by α-T-13′-OH (190 nM), δ-T-13′-OH (540 nM), and δ-T-13′-COOH (2 μM) (Pein et al., 2018). Treatment of activated PMNL with 3 μM LCM effectively blocked 12- and 15-LO product formation, whereas only the 12-LO pathway was blocked by α-5′-T-COOH, α-3′-T-COOH, and γ-3′-T-COOH (Pein et al., 2018). Conversion of arachidonic acid to leukotrienes *via* 5-LO was blocked by δ-T3-13′-COOH (human recombinant enzyme: IC_{50} 35–57 nM) (Richomme et al., 2017; Pein et al., 2018) and 1 μM (Jang et al., 2016); neutrophils (IC 260–345 nM) (Richomme et al., 2017; Pein et al., 2018), whereas product formation

TABLE 5 | Overview on the interference of chromenols with molecular targets and key enzymes connecting inflammation and carcinogenesis.

NF-κB			MAPKs			
Sargachromenol						
TNF-α p65 Trl HUVEC 40 µM inhibition (Gwon et al., 2017)	TNF-α p65 PE HUVEC 40 µM inhibition (Gwon et al., 2017)	LPS p65 Trl BV-2 60 µM inhibition (Kim et al., 2014)	TNF-α IκB-α Phos HUVEC 40 µM inhibition (Gwon et al., 2017)	LPS JNK Phos BV-2 60 µM inhibition (Kim et al., 2014)	LPS p38 Phos BV-2 60 µM no inhibition (Kim et al., 2014)	LPS ERK Phos BV-2 60 µM inhibition (Kim et al., 2014)
TNF-α IκB-α Phos HUVEC 40 µM inhibition (Gwon et al., 2017)	LPS IκB-α Phos BV-2 60 µM inhibition (Kim et al., 2014)					

The content of each cell of the table is constructed as follows (read from top to bottom): (i) used stimulus; (ii) investigated parameter; (iii) cell type tissue, mouse, or other models used for the studies; (iv) used concentration of the respective compound; (v) observed effect on the studied parameter; (vi) reference. The following abbreviations are used. BV-2, brain microglial cells transformed by recombinant retrovirus (v-raf/v-mic); JNK, c-Jun N-terminal kinase; ERK, extracellular-signal regulated kinase; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; p65, nuclear factor NF-κB p65 subunit; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; Phos, phosphorylation; PE, protein expression; Trl, translocation; TNF-α, tumor necrosis factor α.

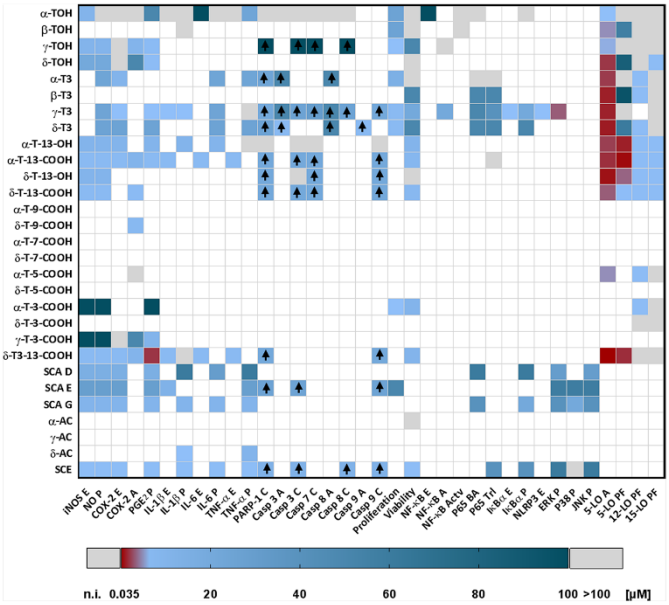


FIGURE 8 | Heatmap illustrating the effectiveness of chromanol and chromenol structures on selected targets. If not indicated otherwise, the plotted effects represent inhibitory effects of the respective compound on distinct parameters; induced parameters are marked with an arrow (↑). The color coding of the presented heat map ranges from high-affinity targets and parameters (effect with <1 µM) presented in red to low-affinity targets and parameters (effects with >1 µM to ≤100 µM) presented in dark blue. If a compound did not affect a specific factor/parameter or showed low effectiveness (>100 µM), the factor/parameter is marked in light gray. Factors and parameters lacking data are marked in white. The heat map is considered as simplified guide for orientation and does not provide a detailed summary of the topic. All concentrations are given micromole (µM). Abbreviations used are: Actv, activation; A, activity; BA, binding affinity; C, cleavage; E, expression; PF, product formation; P, production; T, translocation.

mediated by 12- and 15-LO remained unchanged (Jang et al., 2016). The discrepancy in IC_{50} values in the inhibition of cell-free 5-LO likely depends on the different assay conditions. While Pein et al. analyzed specific 5-LO products by reverse-phase high-performance liquid chromatography with ultraviolet detection, Jang et al. used an indirect colorimetric assay, which determines the formation of hydroperoxides. For SCMs, namely 5′-T-COOH and 3′-T-COOH, no inhibitory effect was observed at the tested concentrations up to 3 μM, except for α-5′-T-COOH (IC_{50} 750 nM) (Pein et al., 2018).

Sargachromanols

Blocking of NF-κB activation with SCAs by inhibiting the phosphorylation of p65 and IκB-α, thereby protecting IκB-α from degradation, has been shown in LPS-activated RAW264.7 macrophages (Heo et al., 2014) and in IL-1β-activated MG-63 osteosarcoma fibroblasts (Yoon et al., 2012b) for SCA D (60 μM) and G (20 μM), respectively. In addition, interference of SCAs D, E, and G with the MAPK pathways, namely phosphorylation of JNK, ERK, and p38, has been observed in LPS-stimulated RAW264.7 macrophages and IL-1β-activated MG-63 osteosarcoma fibroblasts (Yoon et al., 2012b; Lee et al., 2013; Heo et al., 2014).

Chromenols

Like SCAs, δ-SCE has been shown to interfere with the NF-κB and the MAPK pathways. In TNF-α-stimulated endothelial cells (Gwon et al., 2017) and LPS-stimulated microglia cells (Kim et al., 2018), p65 translocation and the phosphorylation of IκB-α were inhibited by 40 μM and 60 μM δ-SCE, respectively. In the same cell models inflammation-induced phosphorylation of JNK and ERK was diminished by δ-SCE, whereas p38 remained unchanged (Kim et al., 2018) (**Table 5**).

LOW AND HIGH-AFFINITY MOLECULAR TARGETS

The heat map in **Figure 8** provides a simplified overview about high- and low-dose bioactivities of the different chromanols and chromenols for a rapid assessment. The selection of compounds and parameters is based on a comprehensive review of the current literature about chromanols and chromenols and focusses on the important biological functions described for these compounds in the context of inflammation and cancer. For reasons of simplification, we did not take into account compound-specific uptake kinetics or cell type- or animal model-specific differences. For more detailed information, the reader is referred to **Tables 1–5** which summarize our current knowledge on the chromanols and chromenols described in the respective sections. For comparison, presented concentrations are IC_{50} values or the lowest reported concentrations affecting the respective parameters.

In the studies considered here, T3s often showed higher effectiveness on the induction or suppression of biological activities linked to inflammation and cancer than TOHs. Furthermore, oxidative modification of the terminal side-chain often substantially increases the anti-inflammatory capacity of respective compounds compared to parental compounds, such as TOHs and T3s. Amplexichromanols, sargachromanols and sargachromenols are also characterized by oxidative modifications of the side-chain, which might rationalize potent interactions with inflammatory targets, which needs further investigation. Notably, regulation of different target genes, proteins, and nuclear receptors can hardly be generalized. For instance, within the group of investigated targets, 5-LO is mostly inhibited by a few compounds, with δ-T3-13′-COOH showing strongest inhibitory effects (IC_{50} 35 nM) and α-TOH showing the least (IC_{50} 1 μM). In contrast, the COX-2-regulated formation of signaling molecules is most efficiently inhibited by γ-T3. In summary, especially 5-LO seems to represent a high affinity (affected at concentrations <1 μM) and therefore specific target for the LCMs of vitamin E. Most of the other observed effects, like mediation of caspase activity, anti-proliferative effects, inhibition of NO formation, are probably the result of a stimulation involving low-affinity targets (affected at concentrations ≥1 μM). However, as implied by the heat map in **Figure 8**, further studies are required for a comprehensive evaluation of the potential of chromanol and chromenol structures to serve as lead structures for the development of future anti-inflammatory therapeutic approaches.

CONCLUSION

For our review, we selected chromanols and chromenols for which data on anti-inflammatory and anti-carcinogenic effects were available in public databases of the scientific literature. The structures of our interests were tocopherols, tocotrienols, and their respective metabolites (which are produced in the liver under physiological and pathophysiological conditions) as well as structurally related compounds including sargachromanols, sargachromenols, and amplexichromanols. Criteria for the evaluation of compounds as possible lead structures for future therapeutic targets were their effects on key inflammatory and apoptotic pathways, proliferation, and interaction with (nuclear) receptor and enzymes that connect inflammation with carcinogenesis. Within this group of selected structures, tocopherols, more precisely α-TOH, are by far the most extensively studied compounds. However, the effects of TOHs are mostly only marginal compared to other compounds described in this review.

It should be noted that the methylation pattern of the chromanol ring system significantly affects inflammation and carcinogenesis. For instance, non-α-TOH and non-α-T3 forms affect eicosanoid- and cytokine-mediated inflammation as well as the cleavage of caspases that mediate apoptosis. Further, T3s are more potent in inhibiting caspase cleavage compared to the respective TOH forms. Tocopherol- and T3-derived metabolites and carboxychromanols more than hydroxychromanols inhibit LO, and in particular 5-LO, effectively and reduce the viability of multiple cancer cell lines. Furthermore, sargachromanols interact

with MAPK and NF-κB pathways, assuming their crosstalk with both, carcinogenesis and inflammation, while sargachromenols mediate anti-carcinogenic effects. Although our knowledge about biological activities of amplexichromanols is sparse, first results indicate their potential for pharmacological applications.

The development of clinically relevant nitric oxide-, eicosanoid-, or cytokine-inhibiting agents or agents that interact with signaling pathways of inflammation is challenging with respect to selectivity and toxicity. Next, although blocking inflammation is meant to be protective, its permanent or long-term inhibition may cause damage to the body (Brasky et al., 2017). Although detrimental effects of naturally occurring chromanols and chromenols cannot be excluded yet, they are less likely for this group of lead compounds in light of the good tolerability of TOHs and T3s at low to moderate doses. Further studies are required to evaluate whether the observed effects of chromanols and chromenols on inflammation and carcinogenesis are indeed beneficial in humans. Until today, no human clinical trials have been published that provide valid information on the biological activity, bioavailability, kinetics, systemic distribution, or local accumulation of these compounds. However, this groups of molecules appears to be promising as lead structures for future anti-inflammatory and/or anti-cancerogenic therapeutic approaches.

LIMITATIONS

Our review is based on a recent systematic review of Birringer et al. (2018), which presented the first comprehensive overview on the diversity of chromanol and chromenol structures and their biological functions. The aim of our review was to more selectively describe the effects on signaling pathways involved in inflammation, apoptosis, cell proliferation, and carcinogenesis and the underlying modes of action for selected chromanols and chromenols. We are aware of the lack of data for a variety of chromenol structures in our overview. We therefore focused on chromanols and chromenols only where adequate data was available that reported anti-inflammatory and anti-carcinogenic properties. For a more detailed description of the structural and chemical properties of all 230 chromanol and chromenol structures, the reader is referred to (Birringer et al., 2018).

AUTHOR CONTRIBUTIONS

MW and SK wrote the manuscript. MW, SK, MS, MB, and SL designed and structured the manuscript, MS, MB, SL, AK, and OW supervised the project and carefully read, evaluated, and discussed the content of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Discussion

Driven by a small community of interested and ambitious scientist, research on vitamin E-derived LCMs has made great progress over the last decade. Nevertheless, the LCMs still represent a small and underestimated area in the broad field of vitamin E research and their proposed significance for human health is controversially discussed. The obvious reason for this is the lack of convincing *in vivo* data confirming the promising results of various *in vitro* studies. However, the resistance of the established vitamin E community against this new interpretation of vitamin E function does also contribute to the 'highly speculative' image of the LCMs.

The current research on the field is predominantly focused on the identification of fundamental biological functions as well as on the elucidation of potential signaling pathways affected by the LCMs. Only a few *in vivo* studies in mice have generated first insights into their potential physiological role. In addition, human studies on the vitamin E-derived LCMs are sparse and limited to the determination of their concentration in blood. Nevertheless, the importance of these initial *in vitro* and *in vivo* studies should not be underestimated, since they enabled (i) the identification of a variety of promising biological functions; (ii) generated hints for LCM accumulation in peripheral tissues and (iii) provided evidence for a systemic relevance of the vitamin E-derived LCMs in the human body. Overall, the mentioned findings offer promising starting points for future experiments.

Based on the objectives mentioned in Section 5 "Aim of the thesis", a number of central questions arose which were experimentally addressed in the enclosed manuscripts. Hence, the discussion about the contribution of these manuscripts to the scientific progress in the field will predominantly focus on the following research priorities (RP):

RP1 Do potential regulatory functions of the LCMs affect crucial factors in the development of atherosclerosis, *i.e.* lipid metabolism of macrophages and inflammation?

RP2 Which signaling pathways, *i.e.* receptors, signaling molecules or enzymes, are affected by the LCMs?

RP3 Are there differences between the efficiency and the mediated effects of the LCMs and their respective vitamin precursors?

8.1 Contribution of the integrated manuscripts to the progress of research in the field of vitamin E-derived LCMs

In line with most of the recent studies in the field, the objectives of this PhD thesis are predominantly settled in the area of basic research. The included manuscripts can be assigned to two different task areas. First, the integrated review articles (manuscripts VI to X) were used to generate a comprehensive overview about the current knowledge on the LCMs to enable the identification of promising research topics and the generation of working hypotheses. Second, the integrated research articles (manuscripts I to IV) were used to

elucidate fundamental biological activities of the vitamin E-derived LCMs α-T-13'-COOH and δ-TE-13'-COOH (GA). The experiments were therefore focused on potential regulatory functions of the compounds on central processes in the development of atherosclerosis on cellular level, *i.e.* foam cell formation and inflammation in macrophages. In contrast, manuscript V presents a hitherto unknown function of α-TOH in preventing cardiac damage after myocardial infarction in mice, which could represent an interesting approach for the therapeutic application of α-TOH. Because of this important finding, the article was included in this PhD thesis although it does not fit with the concept that the LCMs represent the functional molecules of vitamin E. Hence, the contribution of manuscript V to RP1 to RP3 will not be discussed in the section below. A short summary of the most important progressions achieved with the manuscripts I to IV is provided in **Figure 7**.

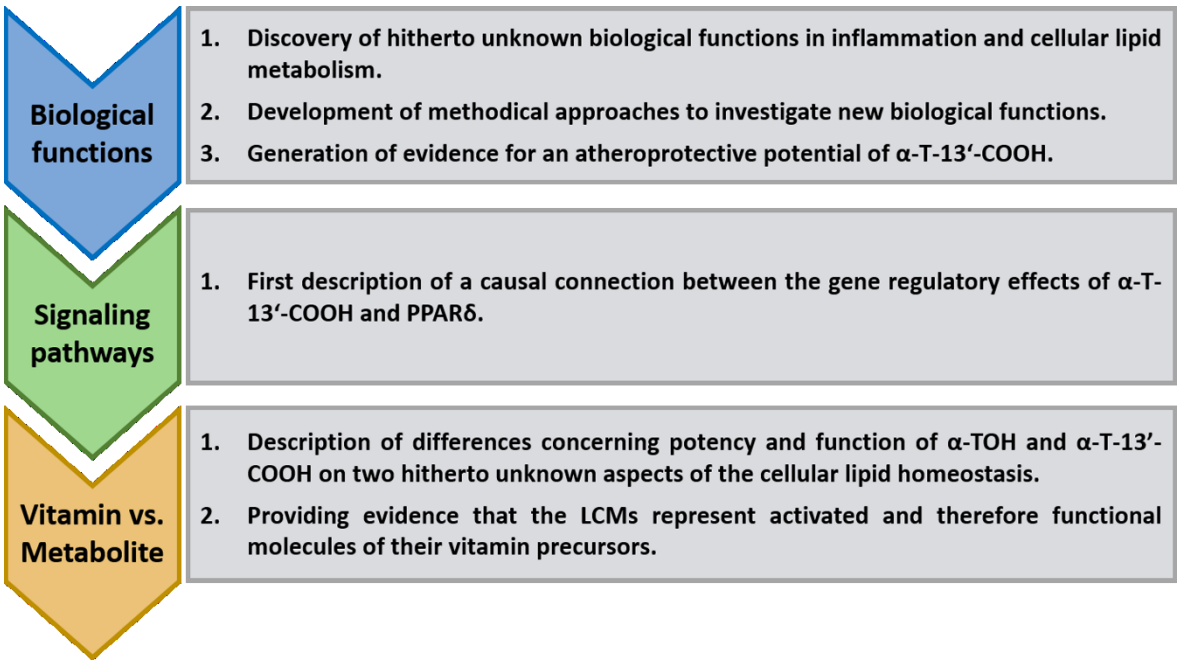

Figure 7: Personal contributions to the progress of research in the field of vitamin E-derived LCMs. *LCM* (long-chain metabolite), *PPARδ* (peroxisome proliferator-activated receptor δ).

8.1.1 Biological functions of the LCMs

8.1.1.1 Modulation of lipid metabolism in macrophages

The investigation of potential regulatory functions of α-T-13'-COOH in the lipid metabolism of macrophages was plausible for several reasons. Most importantly, recent *in vivo* data revealed that α-T-13'-COOH was selectively absorbed by murine peritoneal leukocytes, indicating a preferred uptake of α-T-13'-COOH into immune cells (Pein et al. 2018). Further, a previous investigation of our group provided first evidence for a contribution of the vitamin E-derived LCMs in lipid metabolism, showing that α-T-13'-COOH enhanced the expression of CD36. Surprisingly, the regulation of CD36 had no influence on oxLDL uptake, on the contrary, oxLDL uptake was even diminished by α-T-13'-COOH (Wallert et al. 2014b). Based on these promising

results, manuscript III (under my collaboration) investigated the regulatory effect of α-T-13'-COOH on the expression of PLIN2 – a central mediator of intracellular lipid storage – in human THP-1 macrophages. The investigation showed that α-T-13'-COOH enhanced the expression of PLIN2, which at least in parts protected human THP-1 macrophages from stearic acid induced lipotoxicity (Schmölz et al. 2018). In addition, results from large-scale epidemiological studies provided also evidence for a casual association between TRLs, *i.e.* chylomicrons and VLDL, with macrophage foam cell formation and atherosclerosis. Thus, manuscript I expanded the existing knowledge by the investigation of regulatory effects of α-T-13'-COOH on the expression of LPL – a key enzyme of cellular free-fatty acid (FFA) supply – as well as its physiological regulator ANGPTL4. Interestingly, α-T-13'-COOH did not affect the expression of *Lpl*, but strongly induced the expression of *Angptl*4. In line with this, α-T-13'-COOH efficiently reduced the enzymatic activity of LPL and was therefore able to protect human THP-1 macrophages against excessive lipid accumulation in presence of VLDL oversupply. To enable the determination of neutral lipids and LPL activity in the same cell sample, it was necessary to develop a new methodical approach for a cell culture based real-time fluorescence assay. The method details are explained in manuscript II. To complete the regulatory picture of the LCMs on mechanism involved in macrophage foam cell formation, a running project of our group is currently investigating the effects of α-T-13'-COOH on cellular cholesterol export via ABCA1.

Taken together, the discovered biological activities provide strong evidence for a regulatory role of α-T-13'-COOH in different areas of macrophage foam cell formation, *i.e.* lipid uptake (via CD36 and the LPL system), lipid storage (via PLIN2) and probably lipid export (via ABCA1). Therefore, the contribution of the included manuscripts (created by the author or with his collaboration) to RP1 can be described as follows:

(i) The discovery of two additional biological functions of α-T-13'-COOH, *i.e.* the ANGPTL4-mediated regulation of neutral lipid import and the PLIN2 mediated regulation of lipid storage, significantly improved the existing knowledge about a regulatory role of the LCMs in the lipid metabolism of macrophages.

(ii) The development of a simple and rapid *in vitro* assay for the comprehensive investigation of the LPL system provides a methodical platform for future studies on the LCMs or any other promising compound.

(iii) The linkage of the studies mentioned above together with first impressions from the ongoing project on ABCA1 yield a detailed picture of the regulatory role of α-T-13'-COOH in different key mechanism of macrophage foam cell formation. We therefore hypothesize that α-T-13'-COOH could probably also affect the complex development of atherosclerosis *in vivo*. If so, results of the manuscripts I and III, along with the previous data of Wallert *et al.* (Wallert et al. 2014b) would indicate an atheroprotective potential of α-T-13'-COOH.

8.1.1.2 Modulation of the inflammatory processes

Anti-inflammatory activities are by far the most well investigated part of the LCM function. To date, anti-inflammatory activities of various LCMs have been shown in different *in vitro* and *in vivo* models (the reader is referred to Section 3.3.1 “Anti-inflammatory activit”). In these studies, enzymes, such as iNOS, COX-2 and 5-LO, as well as pro-inflammatory factors, like pro-IL-1β, IL-6 and TNF-α, appeared as major targets for the anti-inflammatory function of vitamin E-derived LCMs (Wallert et al. 2020). Since inflammation also represents a crucial factor in the development of atherosclerosis, anti-inflammatory effects of α- and δ-LCMs were investigated in different types of macrophages (Wallert et al. 2015; Schmölz et al. 2017; Pein et al. 2018).

The contribution of the anti-inflammatory activity of vitamin E-derived LCMs to their proposed systemic relevance is a central topic of our group. Besides their physiological significance, we are also interested in the potential use of LCMs for therapeutic applications and drug development. Unfortunately, this kind of investigations required access to large amounts of the compounds in high purity. To date, most of the prominent vitamin E-derived LCMs, such as α-T-13’-COOH, are not commercially available and must be produced via chemical synthesis. Thus, they do not represent preferred candidates for therapeutic applications so far. However, a common method for drug development is the use of bioactive natural compounds. Interestingly, the seeds of *Garcinia kola E. Heckel*, a plant still used in African ethno medicine, contains high amounts of δ-TE-13’-COOH the so called G. Previous studies on pure GA or extracts from the seeds of G*arcinia kola E. Heckel* already revealed anti-oxidative (Terashima et al. 2002) and anti-proliferative (Mazzini et al. 2009) functions of this promising natural compound. Therefore, manuscript IV (with my collaboration) focused on the investigation of anti-inflammatory effects of GA as well as on an optimization of GA extraction and purification from garcinia kola seeds. First, the efficiency of GA isolation was increased by 6.6-fold compared to already existing protocols. Second, GA reduced the expression of iNOS, COX-2, pro-IL-1β, IL-6 and TNF-α as well as the formation of their respective products, *i.e.* nitric oxide, PGE2, and TXB2, in LPS-stimulated murine RAW264.7 macrophages. In contrast, intraperitoneal application of GA to Apoe$^{-/-}$ mice did not affect inflammatory processes during atherogenesis. Thus, GA seems to be an efficient treatment for acute high-grade inflammation, while it has no effect on chronic low-grade inflammation. The observed anti-inflammatory potential of δ-TE-13’-COOH *in vitro* was confirmed by a simultaneous investigation of a collaborating group, where GA (300 nM) efficiently inhibited PGE2 formation in LPS-stimulated monocytes as well as the activity of isolated 5-LO (Pein et al. 2018).

In summary, manuscript IV together with the results of Pein *et al.* provide evidence for a very potent ant-inflammatory activity of δ-TE-13’-COOH, among others in macrophages. Further, GA could probably serve as an efficient treatment of acute high-grade inflammation. The contribution of manuscript IV (under my collaboration) to RP1 can be described as follows:

(i) The discovery of an anti-inflammatory function of δ-TE-13’-COOH in murine RAW264.7 macrophages significantly expanded the existing knowledge about the biological activities of this promising natural compound and provided evidence for its potential use for pharmacological applications.

(ii) The significant improvement of existing protocols for the extraction and isolation of GA from the seeds of *Garcinia kola E. Heckel* enabled a facilitated access to large amounts of δ-TE-13'-COOH in high purity, paving the way for its potential use in drug development.

(iii) The observation that GA revealed no effect on chronic low-grade inflammation in atherosclerotic mice indicated that although GA represents a potent anti-inflammatory compound, other LCMs (α-T-13'-COOH) are probably more potent *in vivo* (separately discussed in Section 8.2.3 "Utilized compounds").

8.1.2 Affected signaling pathways

Although various biological activities of vitamin E-derived LCMs have been described so far, almost nothing is known about the signaling pathways involved in their regulatory function. In a study on the structure-function relationship of different LCMs, scientists of our group revealed that the effects of the LCMs depend on the presence of their complete molecular structure, *i.e.* chromanol ring system, modified side-chain and functional groups (-OH or -COOH). It was shown that only the LCMs but not their vitamin precursors and other compounds, *i.e.* α-CEHC mimicking the chromanol ring system and pristanic acid imitating the modified side chain, exert specific and distinct effects on the expression of iNOS and CD36. Thus, Schmölz *et al.* suggested the presence of a receptor or a specific signaling pathway mediating LCM function. In line with this hypothesis, Podszun *et al.* described an interaction of α-T-13'-COOH with the nuclear receptor PXR. In their study, α-T-13'-COOH increased the activity of PXR (in a reporter gene assay) as well as the expression of the PXR target gene P-gp in human epithelial-like colon LS180 cells. As a further prove for the interaction of vitamin E-derived LCMs with PXR, a recent preprint showed that PXR activity is also increased by GA (Bartolini et al. 2020).

The elucidation of potential signaling pathways mediating the regulatory function of α-T-13'-COOH on the lipid homeostasis of human THP-1 macrophages was also included in manuscript I. Since ANGPTL4 represents a well-known target gene of PPARδ (Makoveichuk et al. 2012) – the predominant PPAR form in human THP-1 macrophages (Vosper et al. 2001) – the PPARδ antagonist GSK3787 was used to investigate whether the nuclear receptor was involved in the induction of *Angptl4* expression by α-T-13'-COOH. Indeed, co-incubation of α-T-13'-COOH and GSK3787 significantly reduced the induction of *Angptl4* expression, indicating that the gene regulatory function of α-T-13'-COOH is probably mediated by PPARs. This concept seems to be plausible, since other studies also observed an induction of PPAR target genes, such as *Plin2* and *Cd36*, by α-T-13'-COOH (Wallert et al. 2014b; Schmölz et al. 2018). However, inhibition of PPARδ had no effect on functional study parameters, *i.e.* LPL activity and intracellular lipid accumulation. We therefore concluded that the regulatory effects of α-T-13'-COOH might not only result from a specific interaction with a single nuclear receptor.

In summary, manuscript I together with the results of previous studies provide first hints for an interaction of α-T-13'-COOH and other vitamin E LCMs with nuclear receptors, such as PXR and PPARs. Nevertheless, further experiments are necessary to fully unravel the regulatory pathways. The contribution of manuscript I to RP2 can be described as follows:

(i) Manuscript I provides the first description of a causal connection between the inhibition of PPARδ and the subsequent interference with the regulatory effect of α-T-13'-COOH on the PPAR target gene *Angptl4*.

(ii) The discovery of a potential mediation of the gene regulatory function of α-T-13'-COOH by PPARs generates new starting points for future investigations on LCM signaling.

8.1.3 Functional comparison of metabolites and vitamin precursor

Most of the studies on biological activities of vitamin E-derived LCMs also considered the question, whether the LCMs reveal more potent or even different effects than their vitamin precursors. Indeed, differences in potency and modes of action between the two compound classes were discovered for all areas of their known functional spectrum, *i.e.* anti-inflammatory effects (Wallert et al. 2015; Schmölz et al. 2017), anti-carcinogenic effects (Birringer et al. 2010), regulation of lipid metabolism (Wallert et al. 2014b), interaction with pharmaceuticals (Podszun et al. 2017) and regulation of vitamin E metabolism (Torquato et al. 2016a). The key findings of these investigations can be summarized as follows: (i) The potency of the LCMs is generally higher than the potency of their vitamin precursors, *i.e.* significantly lower concentrations of the LCMs are required to reveal regulatory effects. (ii) The 13'-COOH metabolites represent the most potent compounds within the group of vitamin E LCMs. (iii) The LCMs revealed different modes of action compared to their metabolic precursors.

The manuscripts I and III also considered potential differences between efficiency or functionality of α-T-13'-COOH and α-TOH on two new aspects in the regulation of cellular lipid homeostasis. The results of both manuscripts confirmed that significantly lower concentrations of α-T-13'-COOH (5 µM) were required for the regulation of the investigated target genes, *i.e. Angptl4, Lpl* and *Plin2*, compared with α-TOH (100 µM). In addition, manuscript I revealed that α-T-13'-COOH was able to protect human THP-1 macrophages against excessive lipid accumulation in the presence of VLDL oversupply, while α-TOH had no effect. In line with this, manuscript III showed that α-T-13'-COOH damped the negative impact of stearic acid induced lipotoxicity on cell viability of THP-1 macrophages, while α-TOH even increased the cytotoxic effects. Hence, both investigations provided evidence for functional differences in the regulation of the cellular lipid homeostasis between the LCMs and their vitamin precursors. The contribution of the manuscripts I and III to RP3 can be described as follows:

(i) The description of differences concerning potency and function of α-TOH and α-T-13'-COOH in two hitherto unknown aspects of the cellular lipid homeostasis represent a useful expansion to the already existing knowledge on the topic.

(ii) The results of the manuscripts I and III further strengthened the concept that the LCMs represent activated and therefore functional molecules of their vitamin precursors.

8.2 Limitations of current LCM research

The following section will discuss general limitations of the current LCM research as well as specific limitations of the included manuscripts. A short summary of the most important limitations is provided in **Figure 8**.

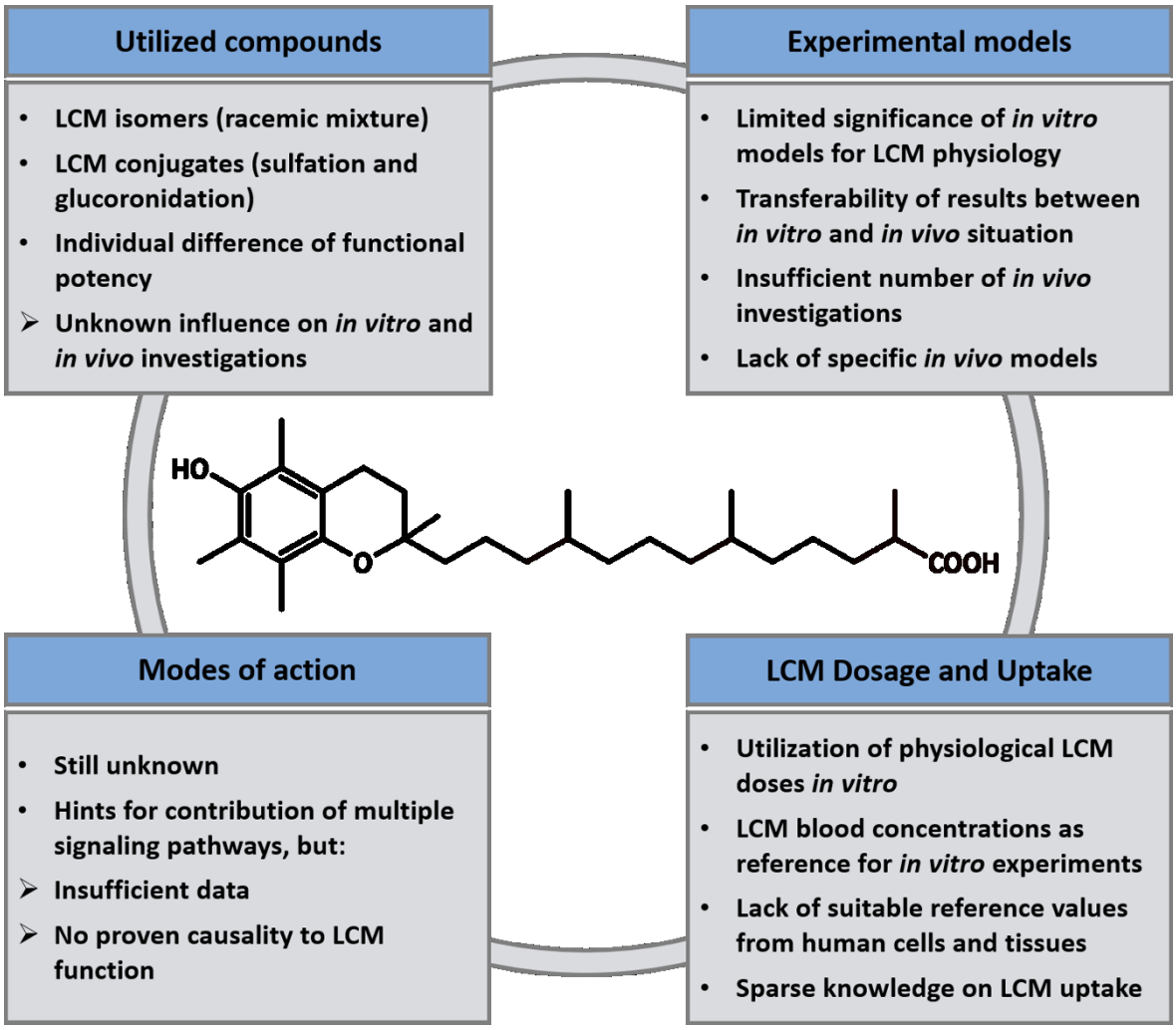

Figure 8: Schematic overview about major limitations of current LCM research. *LCM* (long-chain metabolite).

8.2.1 Utilized experimental models

The choice of a suitable experimental model generally depends on the respective research objectives. In case of the vitamin E LCMs, current research is predominantly focused on the elucidation of biological functions *in vitro*, while the *in vivo* situation plays only a subordinate role (Kluge et al. 2019). This corresponds to the frequency of use of *in vitro* and *in vivo* models in the currently available studies on vitamin E-derived LCMs. Here, cell culture studies represent the predominant test system, while *in vivo* models are poorly used.

The majority of investigations on the LCMs intend to unravel toxic effects, biochemical processes, regulatory functions and differences between the potency of the metabolites.

Hence, cell culture models are used to enable the simplified investigation of specific aspects of the LCM function. In addition, a systemic relevance of the LCMs in the human body was also suggested. However, the complex test environment for the appropriate investigation of potential systemic effects is difficult to imitate *in vitro*. Hence, suitable *in vivo* models are required to investigate the hepatic metabolisation, body distribution and potential physiologically relevant functions of the LCMs.

8.2.1.1 In vitro models

The term '*in vitro* model' represents a collective term for established cell lines, primary cells or stem cells that offer a controlled experimental environment (Alberio et al. 2012). To date, various *in vitro* models, such as human HepG2 liver cancer cells, human THP-1 macrophage-like cells, human HT-29 colorectal adenocarcinoma cells, human HTC 116 colon carcinoma cells, human epithelial-like-colon LS180 cells or murine RAW264.7 macrophages, were used to investigate different functional aspects of the LCMs (Schubert et al. 2018; Wallert et al. 2020). Cellular models are the instrument of choice for the initial characterization of new compounds, because they allow the transfer of complex physiological or pathophysiological processes to simpler molecular ones. Given that the current state of research on vitamin E-derived LCMs is still on the level of basic research, *in vitro* models are useful tools for the generation of preliminary data for more profound investigations. In more detail, cells provide several advantages, such as the ability for a direct exposure to the LCMs; controlled environmental conditions; the ability to augment, block or modulate different cell responses; and the ability to measure cell responses in absence of vitamin E metabolism (Alberio et al. 2012). Nevertheless, the *in vitro* models cannot cover the complex biochemical, physiological or metabolic processes affecting LCM functionality in living organisms. Hence, transferability of results generated *in vitro* to the *in vivo* situation is still a matter of debate. Beside this obvious limitation, *in vitro* models provide further disadvantages, such as the influence of chemical properties on the bioavailability of the applied LCMs, *i.e.* solubility, volatility and precipitation, or chemical interactions altering the culture conditions, *i.e.* pH, osmolarity, protein binding (Nesslany 2017).

The manuscripts I to IV are based on different *in vitro* models, which have the advantages and disadvantages mentioned above. In these studies, the experiments are focused on the investigation of regulatory functions of the LCMs on key factors in the pathogenesis of atherosclerosis, *i.e.* foam cell formation and inflammation. For this kind of experiments, the use of primary human cells as an *ex vivo* model is considered as the gold standard. For example, Pein *et al.* used primary polymorphonuclear leukocytes (PMNL) and peripheral blood mononuclear cells (PBMC) to simulate human-like conditions for the investigation of immunomodulatory functions of α-T-13'-COOH (Pein et al. 2018). However, human primary cells are difficult to handle and their availability is often limited. Therefore, the human-derived leukemia monocytic cell line THP-1 was used for the experiments of the manuscripts I – III. This cell line is commonly used as a replacement of human PBMCs and can easily be differentiated to macrophage-like cells by treatment with phorbol-12-myristate-13-acetate (PMA) (Bosshart und Heinzelmann 2016). Nevertheless, THP-1 cells cannot be completely

equated with PBMCs since both cells revealed differences concerning the degree of gene expression (basal and after stimulation) and the secretion of cytokines. For example, PBMCs show a significantly higher LPS responsiveness compared with THP-1 monocytes, making THP-1 cells a poor replacement model for the investigation of inflammatory response in humans (Chanput et al. 2014). However, none of the described differences between PBMCs and THP-1 cells interferes with the investigated parameters of the manuscripts I to III. Further, the same *in vitro* model was used for a previous study on the regulatory effects of α-T-13'-COOH on macrophage foam cell formation (Wallert et al. 2014b). In my opinion, it was therefore justified to use THP-1 macrophages as an established experimental model that at least in parts allows conclusions to the *in vivo* situation. In addition, THP-1 macrophages also provide several advantages over PBMCs, such as a higher doubling rate, long culture times (25 passages) without changes in cell sensitivity and activity as well as a low variability in the cell phenotype because of the homogeneous genetic background (Chanput et al. 2014).

Manuscript IV investigated the anti-inflammatory effects of GA (δ-TE-13'-COOH) in presence of a LPS stimulus. Therefore, the *in vitro* model had to be switched from human THP-1 macrophage-like cells to murine RAW264.7 macrophages for several reasons: (i) As already mentioned, human THP-1 macrophages represent a poor *in vitro* model for the investigation of LPS response (Chanput et al. 2014). (ii) Human macrophages do not express functional iNOS (Gross et al. 2014), which represented a central investigation target of manuscript IV. (iii) The *in vitro* model should be used for the generation of preliminary data for a subsequent investigation in mice. Hence, the utilized *in vitro* model should predominantly allow for drawing conclusions to the *in vivo* situation in mice. Murine RAW264.7 macrophages represent an established cell line that is commonly used for the investigation of anti-inflammatory effects of various compounds against a LPS stimulus (Kumar R und Abraham 2017; Lee et al. 2016). Therefore, the use of RAW264.7 macrophages seemed reasonable, since previous studies of our group also relied on this model (Wallert et al. 2015).

8.2.1.2 In vivo models

In vivo models, such as mice, rats, hamsters or rabbits, represent the preferred tool to generate deeper insights into the interactions of a test compound with a complex physiologic environment. This does among others include the provision of information about interactions with the xenobiotic metabolism as well as on interactions between different cell types or tissues (Tice et al. 2013). Nevertheless, the use of animals as a model for human physiology is not without controversy, since especially rodents – the most frequently used *in vivo* models – show remarkable physiological and genetic differences to humans. Thus, transferability of data generated in these models to the physiological situation in humans is always a matter of debate (Perlman 2016; Shanks et al. 2009). However, *in vivo* models are of special interest for LCM research, since the metabolites are physiologically formed in the liver. Different investigations concerning the determination of LCM blood concentrations in humans already provided evidence for a systemic relevance of these compounds (Wallert et al. 2014b; Ciffolilli et al. 2015; Giusepponi et al. 2017), *i.e.* the metabolites may be transferred to peripheral tissues where they probably serve as regulatory molecules (Schubert et al. 2018).

Interestingly, the hepatic metabolism of vitamin E in rodents is very similar to the one in humans, making them a good model to investigate physiologic effects of the vitamin E LCMs (Wallert 2014a).

Current *in vivo* studies on vitamin E-derived LCMs are predominantly focused on the impact of specific functional aspects (discovered *in vitro*) on the development and the progression of different diseases. Hence, the used *in vivo* models are not specifically designed for vitamin E research and rather represent established and reliable models of human diseases with an assumed connection to one or more of the discovered LCM functions. In addition, the sparse knowledge on the LCM physiology, *e.g.* rate of metabolic elimination, further complicates the choice of a suitable test compound, the form of compound administration and the sufficient dosage of the compounds. In manuscript IV used Apoe$^{-/-}$ mice were used to investigate the impact of GA (δ-TE-13'-COOH) on the progression of atherosclerosis. The mice received 1 mg GA/kg body weight via weekly intraperitoneal injection. Surprisingly, administration of GA had no effect on inflammation during atherogenesis (Wallert et al. 2019). The authors concluded that a model of chronic low-level inflammation, such as atherosclerotic mice, might not be the optimal test system for the pharmacological application of GA. However, it cannot be excluded that factors such as the form of compound administration as well as metabolic elimination of GA biased their results. With respect to these limitations, a recent study provided potential starting points for the improvement of future *in vivo* investigations. Based on their previous *in vitro* data, Pein *et al.* investigated the inhibitory effect α-T-13'-COOH and δ-TE-13'-COOH on 5-LO activity in a zymosan-induced peritonitis model. For this, CD-1 mice received 10 mg/kg of either α-T-13'-COOH or δ-TE-13'-COOH via intraperitoneal injection. Surprisingly, only α-T-13'-COOH but not δ-TE-13'-COOH inhibited 5-LO product formation in leukocytes isolated from exudates, although each compound had efficiently blocked 5-LO product formation *in vitro*. These results for the first time indicated that the form of the administrated LCMs could influence the experimental outcomes *in vivo* (separately discussed in Section 8.2.3 "Utilized compounds"). In addition, another group of mice received 50 mg α-TOH/kg body weight via perioral administration, representing a more physiologically approach of compound administration compared with direct intraperitoneal injection. Despite of this indirect administration, ultra-performance liquid chromatography ESI tandem mass spectrometry analysis revealed that α-T-13'-COOH was accumulated in leukocytes isolated from exudates. This results for the first time indicated that orally administrated α-TOH is metabolized to α-T-13'-COOH in the liver, which is subsequently transferred to peripheral immune cells (Pein et al. 2018). Hence, oral administration of the vitamin precursors could be used for a better simulation of the physiologic situation in future *in vivo* investigations, at least for α-T-13'-COOH.

In summary, the amount of available data on the physiology of vitamin E LCMs is still too small to generate a final blue print for the optimal design of *in vivo* experiments. However, the comparison of both *in vivo* studies mentioned above provides important findings for the improvement of future *in vivo* investigations. (i) There is evidence for a selective physiological relevance of the α-LCMs, which should be taken into account for the choice of test

compounds. (ii) The possibility of a quick metabolic elimination of the test compounds must be considered for their administration and their dosage. (iii) The relevance of available *in vitro* data for future *in vivo* investigations should be carefully reevaluated based on the new insights from both investigations.

8.2.2 Dosage and uptake of the LCMs

Dosage and uptake of the administered LCMs are two important experimental factors with major influence on the accuracy of data generated by *in vitro* and *in vivo* investigations. Because most of the currently available studies on the LCMs (including manuscripts I and III) are based on cell culture models, the following section will predominantly discuss the significance of LCM dosage and uptake for *in vitro* experiments. Nevertheless, both factors are also important for *in vivo* investigations.

One of the major aims of LCM research is to proof the suggested physiologically relevance of the compounds. In theory, determination of physiologic LCM concentrations in cells and tissues of the human body would provide important reference values for the correct dosage of the metabolites *in vitro*. However, low nanomolar concentrations determined in human blood represented the only reference for the assessment of suitable LCM concentrations for *in vitro* experiments until recently (Wallert et al. 2014b; Giusepponi et al. 2017). For example, the mean α-T-13'-COOH plasma concentration of ten healthy volunteers was determined between 26.2 ± 13.9 nM (Pein et al. 2018). In contrast, most of the current *in vitro* studies (including manuscripts I and III) used a α-T-13'-COOH concentration of 5 µM, *i.e.* a supraphysiologic concentration in relation to nanomolar blood levels. The use of supraphysiologic concentrations might represent a common practice for the elucidation of general modes of action of the LCMs, but is in conflict with the proposed physiological relevance of the metabolites. This example illustrates an often-criticized issue in LCM research that is expanded by the lack of data on the cellular uptake of the metabolites. However, this criticism is not fully justified, because current *in vitro* investigations (including manuscripts I and III) did not attempt to imitate the physiologic environment of human blood rather of specific human cells or tissues, such as macrophages or the liver (Birringer et al. 2010; Wallert et al. 2014b; Schmölz et al. 2018). Thus, human blood may therefore not represent the optimal reference matrix for the evaluation of LCM concentrations.

Interestingly, a recent study in mice addressed most of the issues mentioned above. Under basal conditions, murine plasma levels of α-T-13'-COOH (10 - 35 nM) were comparable to the plasma levels in humans (8 - 49 nM) (Pein et al. 2018). Surprisingly, oral application of 50 mg α-TOH/kg body weight had no influence on α-T-13'-COOH plasma concentration, but significantly increased the concentration of the metabolite in exudates (basal: 150 nM; α-TOH treatment: 230 nM) and peritoneal leukocytes isolated from these exudates (basal: 5 nM; α-TOH treatment: 10 - 20 nM). Further, parallel ingestion of α-TOH (50 mg/kg body weight) and sesamin – a known inhibitor of hepatic vitamin E catabolism that blocks CYP4F2/CYP3A4-dependent ω-hydroxylation – only decreased α-T-13'-COOH levels in plasma, while the concentration in exudates remained unchanged (Pein et al. 2018). These results indicate that

blood seems only to serves as a transport matrix for α-T-13'-COOH, while higher amounts of the metabolite accumulate at its potential sites of actions, *e.g.* immune cells. These peripheral long-term stores are only subjected to slight fluctuations and can be increased by the additional administration of α-TOH. Since the physiologic environment of rodents differs from that of humans, Pein *et al.* also proofed their findings in a human matrix, *i.e*. PMNLs isolated from human blood. Interestingly, the basal concentration of α-T-13'-COOH in PMNLs was already on micromolar level (Pein et al. 2018). These results showed that human peripheral cells can accumulate α-T-13'-COOH in significantly higher amounts compared with the low nanomolar levels in blood. In addition, incubation of the PMNLs with 150 nM α-T-13'-COOH for 20 minutes significantly enhanced the intracellular concentration of the metabolite (up to 50 µM), indicating a highly efficient uptake of α-T-13'-COOH.

Taken together the study of Pein *et al.* provides several important findings that should be considered for the assessment and discussion of LCM concentrations in current *in vitro* experiments. First, the LCM blood levels represent a poor reference to derive suitable concentrations for *in vitro* experiments, since peripheral cells and tissues seem to accumulate higher amounts of the LCMs (up to 50 µM of α-T-13'-COOH in human PMNLs). Hence, the LCM concentrations used for current *in vitro* investigations (including manuscripts I and III) would approximately correspond to the physiological situation, at least for α-T-13'-COOH. However, the studies in manuscripts I and III were carried out in human THP-1 macrophages-like cells. Therefore, the determination of α-T-13'-COOH levels in human PBMCs would also be of interest for a more accurate assessment of the physiologic α-T-13'-COOH concentration in macrophages. Second, the results of Pein *et al.* indicate a highly efficient uptake of α-T-13'-COOH into PMNLs. However, uptake kinetics could also differ between primary cells and cell lines. Experiments of our group (unpublished data) generated a first impression on the uptake of α-T-13'-COOH into human THP-1 like macrophages. After 24 h incubation with 5 µM α-T-13'-COOH, the THP-1 macrophages revealed an intracellular concentration of 4 µM of the compound. These results provide evidence for the uptake of α-T-13'-COOH into THP-1 macrophages, although uptake efficiency was significantly lower compared with human PMNLs. Nevertheless, after an incubation time of 24 h (like in most *in vitro* experiments, including that in manuscripts I and III) nearly the complete amount of the administrated α-T-13'-COOH could be detected inside the cell. Third, the findings of Pein *et al.* should be used as a starting point for the establishment of a general database for LCM concentrations in cells, tissues and other matrices with relevance for the LCM function *in vivo*. Broad knowledge on the distribution of the LCMs as well as on their concentration in different *in vivo* matrices could also improve the accuracy and significance of *in vitro* investigations on physiologically relevant LCM functions. Based on our current knowledge on vitamin E storage, metabolisation and accumulation, an initial determination of α-T-13'-COOH concentrations in adipose tissue and muscle (Bjørneboe et al. 1990), liver (Bardowell et al. 2012a), heart (Mustacich et al. 2007) and brain (Fukui et al. 2015) could be of interest.

8.2.3 Utilized compounds

In general, experiments concerning the biological activity of certain compounds highly depend on their purity to ensure that potential effects are contributed to the compound itself and not to side products or contaminations. The vitamin E-derived LCMs (at least the α- and δ-forms) are generated via semi-synthesis from GA (δ-TE-13'-COOH) and thus their purity must be confirmed by GC-MS or HPLC-MS analysis. The purity of the synthesized LCMs used for *in vitro* studies (including that in manuscripts I, III and IV) was determined as > 95% (Birringer et al. 2010; Schmölz et al. 2017) and does therefore meet the quality requirements for pharmaceutical compounds (Singh et al. 2018). In addition, isolated GA was characterized as enantiopure, providing optimal chemical conditions for the semi-synthesis of α- and δ-LCMs (Mazzini et al. 2009). Nevertheless, both LCM forms are obtained as a racemic mixture after their semi-synthesis from GA (unpublished data). This is in contrast to natural vitamin E forms that occur as a single enantiomer (Brigelius-Flohé und Traber 1999). In turn, natural vitamin E-derived LCMs are also considered to be present as one enantiomeric form, indicating that the use of synthetic LCMs for *in vitro* experiments would not correspond to the physiologic situation in humans. However, given that the observed biological effects of the LCMs *in vitro* are probably only attributed to one enantiomeric form, significantly lower concentrations of the relevant enantiomer would be necessary to mediate the observed regulatory effects. Unfortunately, no synthetic approach for the enantiopure synthesis of the respective LCMs is currently existing and it is therefore not possible to prove this hypothesis. Interestingly, a recent analysis of α-T-13'-COOH serum concentrations of healthy volunteers who received 1000 IU *RRR*-α-TOH per day (for one week), revealed three hitherto unknown compounds with identical masses like α-T-13'-COOH. Additional characterization by high-resolution mass spectrometry revealed that these compounds are potential isomers of α-T-13'-COOH. Hence, α-T-13'-COOH blood concentrations are probably detected as a bulk parameter comprised of different isomers (Giusepponi et al. 2017).

Another general issue concerning form and shape of probably all vitamin E-derived LCMs is their chemical modification. Enzymatic conjugation, *i.e.* sulfation and glucuronidation (Freiser und Jiang 2009; Zhao et al. 2010), of the LCMs occurs during the hepatic metabolism of vitamin E and seems to be therefore of relevance for *in vivo* studies. In contrast, the synthetic LCMs used for the majority of *in vitro* studies (including manuscripts I and III) are unconjugated. Investigations in human blood revealed that α-T-13'-COOH and α-T-13'-OH physiologically occur in their conjugated form, *i.e.* as sulfated or glucuronidated conjugates (Wallert et al. 2014b; Ciffolilli et al. 2015; Giusepponi et al. 2017). Interestingly, conjugates of γ- and δ-metabolites were also detected in A549 cells, a cell line that is able to generate vitamin E-derived LCMs via endogenous synthesis (Jiang 2007). Thus, modification of the LCMs seems also to occur in several cell culture models. So far, no comparable results have been described for THP-1 like macrophages, indicating that enzymatic conjugation had no influence on the *in vitro* experiments included in manuscripts I and III. Nevertheless, enzymatic modification of the metabolites could represent an important factor that needs to be considered for future experiments, especially since Jiang *et al.* provided evidence that the sulfated metabolites do

not reveal biological activity (Jiang 2008). Hence, synthetic LCMs used for *in vitro* investigation would always be present in their active (unconjugated) form, while the majority of physiologically LCMs *in vivo* is comprised of inactive conjugates. If this holds true, the transferability of results concerning the biological activity of LCMs *in vitro* to the physiological situation *in vivo* would be even more complicated.

Beside these general factors affecting all LCM forms, individual differences between the compounds are also of interest. To date, a variety of biological activities for different forms of vitamin E-derived LCMs was described. Here, especially the efficacy appeared to differ between the compounds. Although none of the currently available studies directly compared the efficacy of all LCM forms, the entirety of *in vitro* investigations allows for derivation of general structure specific aspects of the LCM function. First, the 13'-COOH metabolites seem to represent the most potent forms among the group of vitamin E-derived LCMs (Wallert et al. 2014b; Schmölz et al. 2017; Wallert et al. 2015; Birringer et al. 2010). Second, there are a few hints that TE-derived metabolites act more potent than the TOH-derived metabolites, at least in cell lines or cell free experimental models (Wallert et al. 2019; Pein et al. 2018). Third, methylation pattern of the chromanol ring of the different LCM forms is probably not as relevant for their effectiveness as it is for their TOH or TE precursors (Schmölz et al. 2017). Interestingly, potency of the different LCM forms seems also to depend on the utilized test system. This topic was recently addressed by an investigation comparing the ability of α-T-13'-COOH and δ-TE-13'-COOH to inhibit 5-LO activity in different experimental models. Initially, the activity of human recombinant 5-LO (pure cell-free enzyme) was inhibited by δ-TE-13'-COOH at an IC_{50} of 35 nM, while almost ten times of the amount was necessary for α-T-13'-COOH (IC_{50} 270 nM). However, analysis of 5-LO activity in primary human PMNLs revealed contrary results. Here, α-T-13'-COOH (300 nM) reduced 5-LO product formation by 80%, while δ-TE-13'-COOH (300 nM) only reached a blocking rate of 50%. These results were confirmed in a murine zymosan-induced periodontitis model, where mice were treated (i.p.) with 10 mg/kg body weight of both compounds. Interestingly, only α-T-13'-COOH and not δ-TE-13'-COOH reduced 5-LO product formation *in vivo*, which was verified by the detection of reduced amounts of 5-LO products (*e.g.* LTC4) in exudates. Additional experiments revealed that both, primary human PMNLs and peritoneal leukocytes isolated from murine exudates, accumulated significantly higher amounts of α-T-13'-COOH, while the amounts of δ-TE-13'-COOH was substantially lower (PMNLs) or even not detectable (peritoneal leukocytes). The authors concluded that the high inhibitory potential of α-T-13'-COOH on 5-LO activity *ex vivo* and *in vivo* was linked to the efficient uptake and specific accumulation of the compound in primary human PMNLs and peritoneal leukocytes compared with the less efficient uptake by or complete absence of δ-TE-13'-COOH in their cells. The results further indicate that although multiple LCMs (including δ-TE-13'-COOH) were able to inhibit 5-LO, only α-T-13'-COOH seems to be relevant for systemic effects *in vivo*. It can therefore be speculated, whether α-T-13'-COOH (key study target of manuscripts I and III) may represent the only suitable LCM for future *in vitro* and *in vivo* investigations on the potential systematic relevance of the vitamin E metabolites.

8.2.4 Modes of action

The elucidation of the exact modes of action underlying the biological effects of vitamin E-derived LCMs represents a central priority of current LCM research, as well as a crucial requirement for future scientific progress on the field. However, the origin of the question concerning the existence of specific regulatory pathways triggered by vitamin E or its metabolites already goes back to the initial discovery of gene regulatory functions of α-TOH in 1988 (Mahoney und Azzi 1988). Since then, various biological activities of the LCMs have been described, but still almost nothing is known about their exact modes of action. Nevertheless, especially specific gene regulatory effects of α-T-13'-COOH, *i.e.* regulation of *iNos*-, *Cox-2*-, *Cd36*-, *Plin2*-, *Angptl4*- and *Il*-expression, provided useful hints on the potential involvement of central signaling pathways linked to these target genes (manuscripts I and III; (Wallert et al. 2014b; Wallert et al. 2015)). Further, a recent *in vitro* study was intended to identify the parts of the LCM molecule that are relevant for the mediation of the observed regulatory functions. The study revealed that the combination of three structural elements of the LCM molecule, *i.e.* chromanol ring, aliphatic side chain and functional hydroxylic- or carboxylic group, seems necessary to exert gene regulatory functions. In contrast, a single-, *i.e.* α-CEHC (chromanol ring) and pristanic acid (side chain), or the combination of only two structural elements of the molecule, *i.e.* α-TOH and δ-TOH (missing functional group), were not sufficient. Based on these results, Schmölz *et al.* suggested the existence of a receptor or specific signaling pathways mediating LCM functions (Schmölz et al. 2017).

If this concept holds true, three different options seem conceivable (The following hypotheses are be based on the current knowledge on α-T-13'-COOH, since almost nothing is known about the other LCMs). First, a peripheral or integral receptor located at the cell surface or within the membrane may exists that binds the LCMs and mediates further signaling. This seems plausible, since it would represent the easiest way of an interaction between the metabolites discovered in blood circulation and peripheral cells (Giusepponi et al. 2017). However, the accumulation of α-T-13'-COOH in peritoneal leukocytes of α-TOH fed mice is contradictory to an exclusive signaling via surface receptors (Pein et al. 2018). Interestingly, α-T-13'-COOH was the only LCM accumulating in these cells, although increased blood concentrations of α-T-11'-COOH and α-T-9'-COOH were detected (Pein et al. 2018). Thus, the selective uptake of α-T-13'-COOH provides evidence for the existence of a specific transport/uptake system, indicating that α-T-13'-COOH must get inside the cell to mediate its regulatory effects. Second, the LCMs could serve as ligands for nuclear or cytosolic receptors that potentially mediate their gene regulatory functions, as it was already described for the metabolites of vitamins A (Perlmann und Jansson 1995) and D (Pike und Meyer 2010). Third, the regulatory functions of the LCMs could be mediated by a combination of downstream signaling pathways from extracellular and intracellular receptors. The following selection of potential signaling pathways mediating regulatory effects of α-T-13'-COOH are predominantly based on current findings of our group (including unpublished data). It should be noted that the mentioned signaling pathways are only suggestions, which need to be carefully validated by further

experiments. Nevertheless, the provided information could serve as a useful starting point for future investigations on LCM signaling.

8.2.4.1 The mitogen-activated protein kinase pathway

The mitogen-activated protein kinase (MAPK) pathway, with its central enzymes extracellular signal-regulated kinase (ERK)1/2, mitogen-activated protein kinase p38 (p38) and c-Jun N-terminal kinase (JNK), represents a signaling cascade that is able to transmit an extracellular signal to the nucleus. Within this cascade, the extracellular signals are translated into gene regulatory signals, enabling a quick and efficient cellular response to various stimuli (Soares-Silva et al. 2016). The cascade is initiated by the binding of a distinct signaling molecule to a surface receptor that is linked to the small G protein rat sarcoma (Ras). After binding of the signaling molecule, Ras is activated by a conformational switch of its active site, which in turn leads to the subsequent activation of rapidly accelerated fibrosarcoma (Raf). After these initial events, the catalytically inactive MAPKs, *i.e.* ERK1/2, p38 and JNK are activated by multiple phosphorylation events, enabling transcriptional regulation of various target genes (Soares-Silva et al. 2016). Experiments of our group revealed that α-T-13'-COOH leads to an increased phosphorylation of ERK1/2 and p38 in murine RAW264.7 macrophages and human fibroblasts already 30 min after compound administration (unpublished data). Interestingly, α-T-13-COOH treatment also increased the expression of dual *specificity protein phosphatase* 1 (*Dusp1*) – an enzymes that deactivates MAPKs by dephosphorylation (Escudero et al. 2019) – at later time points (unpublished data). Although there is currently no proof that blockage or activation of the MAPK pathway affects the expression of genes regulated by α-T-13'-COOH, the results mentioned above provide evidence that the regulatory functions of the metabolite could be at least in parts mediated by the MAPK pathway.

8.2.4.2 The nuclear factor 'kappa-light-chain-enhancer' of activated B-cells pathway

The transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) is a key regulator of the cellular response against stress, including cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens (Bhatt und Ghosh 2014). Under basal conditions, NF-κB remains at its inactive state, *i.e.* bound to the inhibitor of κB (IκB). The IκB protein prevents translocation of NF-κB to the nucleus by masking its nuclear localization signals. Due to the stimulation of different surface receptors, such as toll-like receptors, NF-κB is among others activated via signal-induced degradation of IκB proteins by IκB kinases. After the removal of IκB, the NF-κB subunit p65 is able to enter the nucleus where it induces the expression of specific target genes (Bhatt und Ghosh 2014). Experiments of our group showed that α-T-13'-COOH itself induced the expression of NF-κB target genes. In turn, pre-treatment with α-T-13'-COOH damped the cellular response against LPS, probably because of an adaptive response against the initial α-T-13'-COOH stimulus (unpublished data). Further, the initiation of an adaptive response against α-T-13'-COOH was partially prevented by treatment with a specific inhibitor against the translocation of the p65 subunit (unpublished data). These data indicates that especially the anti-inflammatory effects of α-T-13'-COOH are potentially mediated via the NF-κB pathway.

8.2.4.3 Ion channel mediated signaling pathways

The transmission of extracellular signals via voltage- or ligand-gated calcium channels represents another possibility to mediate intracellular processes. Inflowing Ca^{2+} predominantly exerts regulatory effects on different enzymes by serving as an important co-factor for their activity (Ahvazi et al. 2003; Lee et al. 2007). Nevertheless, gene regulatory effects of Ca^{2+} have also been described (Barbado et al. 2009; West et al. 2001). Experiments of our group showed that the blockage of L-type calcium channels by nifedipine – a well-investigated Ca^{2+} channel blocker – reduced the inhibitory effect of α-T-13'-COOH on ABCA1 mRNA and protein expression (unpublished data). These results were further confirmed by the use of BAPTA-AM, a Ca^{2+} chelator that was specifically designed to act inside the cell. Hence, interception of the inflowing Ca^{2+} and the already present Ca^{2+} inside the cell also reduced the inhibitory effect of α-T-13'-COOH on ABCA1 expression (unpublished data). In addition, inhibition of calpain – a calcium dependent enzyme responsible for the degradation of ABCA1 – reduced the inhibitory effect of α-T-13'-COOH on ABCA1 protein expression. Overall, α-T-13'-COOH could potentially induce Ca^{2+} inflow via interaction with membrane associated calcium channels, leading to transcriptional and post-translational regulation of various targets. However, the involvement of Ca^{2+} in the regulatory function of α-T-13'-COOH needs further confirmation, since only one target protein (ABCA1) was studied so far.

8.2.4.4 Peroxisome proliferator-activated receptor mediated signaling

The PPARs, *i.e.* PPARα, PPARβ/δ and PPARγ, predominantly act as sensors for fatty acids and fatty acid-derived metabolites and can therefore be activated by a variety of molecules (Lefebvre et al. 2006; Neels und Grimaldi 2014; Tontonoz et al. 1998). Based on their function as transcription factors, PPARs are able to regulate the expression of different genes involved in lipid metabolism, inflammation, proliferation and differentiation (Varga et al. 2011). The most common model for PPAR-dependent gene regulation includes the direct transcriptional regulation by heterodimerization of a PPAR isoform with the retinoid X receptor (RXR). Under basal conditions, the heterodimer is bound to the so-called PPAR response element in the promoter region of target genes as well as to a corepressor complex preventing transcription. Upon ligand binding, the corepressor complex is replaced by coactivators, resulting in the transcription of target genes (Varga et al. 2011). Based on their structural similarities to unsaturated fatty acids, *i.e.* natural ligands of the different PPAR forms, the vitamin E LCMs were also suggested as potential PPAR ligands. Hence, the α-T-13'-COOH dependent regulation of the PPAR target gene *Angptl*4 was extensively studied in THP-1 macrophages (manuscript I). Since PPARδ represents the predominant PPAR form in human THP-1 macrophages (Vosper et al. 2001), the chemical PPARδ antagonist GSK3787 was used to investigate whether PPARδ was involved in the induction of ANGPTL4 mRNA expression by α-T-13'-COOH. Indeed, co-incubation of α-T-13'-COOH and GSK3787 significantly reduced the induction of *Angptl*4 expression, indicating that the gene regulatory function of α-T-13'-COOH is probably mediated by PPARs. This concept seems plausible, since other studies also observed α-T-13'-COOH induced induction of PPAR target genes, such as *Plin*2 and *Cd36* (Wallert et al., 2014b; Schmölz et al., 2018). Although inhibition of PPARδ had no effect on

functional study parameters, these results provide nonetheless evidence that the regulatory functions of α-T-13'-COOH could be at least in part mediated by PPARs.

8.2.4.5 Pregnane-X-receptor mediated signaling

The pregnane-X-receptor represents another nuclear receptor with a proven association to α-T-13'-COOH mediated gene expression. Transcriptional regulation via PXR is accomplished by the same mechanisms described for the PPARs, *i.e.* dimerization with RXR and subsequent activation of target gene transcription via ligand binding. Using a reporter gene assay, Podszun *et al.* showed that α-T-13'-COOH was able to induce PXR activity. For further verification, the expression of PXR target genes was investigated in human epithelial-like colon LS180 cells after α-T-13'-COOH treatment. In line with the results of the reporter gene assay, P-gp protein expression was also induced by α-T-13'-COOH (Podszun et al. 2017). In addition, δ-TE-13'-COOH (*i.e.* GA) was also proposed as a selective PXR agonist (Bartolini et al. 2020). With PXR, another nuclear receptor is probably involved in the regulatory functions of vitamin E-derived LCMs, making it more and more likely that these molecules could serve as ligands for nuclear receptors.

8.3 Future perspectives of LCM research

After a decade of research on the vitamin E-derived LCMs, their exact mode of action as well as their significance for human health are still far from being unveiled.

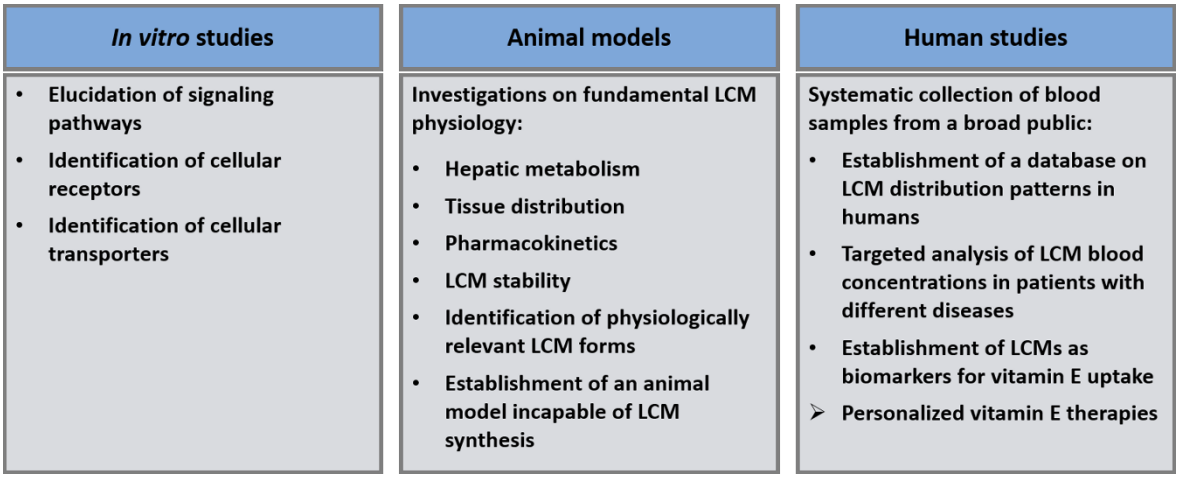

Figure 9: Schematic overview on major aims of future LCM research. LCM (long-chain metabolite)

However, *in vitro* studies discovered a variety of biological functions of the LCMs (Schubert et al. 2018) that were supplemented by initial findings from *in vivo* trials, such as the appearance of LCMs in human blood (Wallert et al. 2014b) or the selective accumulation of α-T-13'-COOH in murine peritoneal leukocytes (Pein et al. 2018). Altogether, these studies contributed to the generation of fundamental hypotheses in the field: (i) The LCMs possess a physiologically relevance for the human body; (i) The LCMs represent functional and therefore active forms of their vitamin precursors; (iii) Essentiality of vitamin E for human health is closely linked to

the LCMs (Galli et al. 2017). As mentioned before, these hypotheses are predominantly based on *in vitro* data that requires careful validation in animal models and humans to change the current picture of vitamin E-derived LCMs as a 'highly speculative area' of vitamin E research. Thus, it seems unavoidable for the scientific progress in the field to switch the focus from *in vitro* to *in vivo* models and human trials. Findings from these 'next generation' studies and trials could generate useful information on the essentiality of vitamin E and it's LCMs for human health as well as on potential clinical benefits of these molecules. The following section will discuss ideas for future *in vitro*-, *in vivo* model based- and human investigations on vitamin E LCMs, which I personally consider as major tasks for the next decade of LCM research. A short summary of the from my point of view most important aims of future LCM research is provided in **Figure 9**. However, the crucial prerequisite for this is to overcome the limitations mentioned in the Section 8.2 "Limitations of current LCM research".

8.3.1 *In vitro* studies

Although *in vitro* models proved their value as an easy accessible, easy to handle and reliable tool for the initial characterization of LCM functions, current research issues concerning physiologic aspects revealed the limitation of these experimental models. Nevertheless, cell culture based studies will still be of interest for future investigations on the general modes of action of the vitamin E-derived LCMs. This includes the elucidation of signaling pathways that mediate LCM functions as well as the identification of proteins involved in their cellular uptake. Here, target-fishing experiments, *i.e.* incubation of immobilized LCMs with cell lysates, could serve as a useful tool for the elucidation of specific LCM-protein interactions. After their separation by 2D gel electrophoresis, the proteins could be isolated and analyzed via MS to identify potential receptors or transporters of the LCMs. In addition, target-fishing experiments should be supplemented by the selective knockdown or overexpression of key enzymes in central signaling cascades as well as by the use of selective chemical inhibitors or activators against signaling proteins. The combination of both methods could potentially enable the identification of fundamental modes of actions in LCM signaling.

8.3.2 *In vivo* studies based on animal models

A recent investigation of Pein *et al.* provided a first impression of the great potential of animal models for future LCM research (Pein et al. 2018). In general, animal models could be used for all kinds of investigations that cannot be carried out in humans or with human material (for ethical reasons). From my personal point of view, future animal studies should therefore predominantly focus on the elucidation of the currently unknown LCM physiology. This includes regulation of vitamin E metabolism, transport mechanisms for vitamin E-derived LCMs in the body, distribution and uptake kinetics of the LCMs in extrahepatic tissues, identification of physiologically relevant forms of vitamin E LCMs as well as their stability against xenobiotic degradation. To accomplish these complex objectives, different methodical approaches are conceivable.

8.3.2.1 Investigation of hepatic metabolism

Since the hepatic metabolism of vitamin E in humans and rodents appeared to be very similar (Wallert 2014a), Cyp4f14 (= CYP4F2) or Cyp3a11 (= CYP3A4) knockout mice, *i.e*. rate limiting enzymes of vitamin E catabolism, could serve as suitable models for the investigation of the physiologically significance of vitamin E and its LCMs (Bardowell et al. 2012b; van Herwaarden et al. 2007). To date, only one study used Cyp4f14$^{-/-}$ mice fed with a soybean oil diet to investigate metabolization rates of α-, γ- and δ-TOH. Interestingly, whole-body metabolization of γ-TOH was reduced by 90% but only by 68% for α- and δ-TOH (Bardowell et al. 2012b). The authors concluded that although Cyp4f14 seems to be the major vitamin E ω-hydroxylase in mice, other CYPs, such as Cyp3a11 are also important for vitamin E metabolism. However, there are currently no studies available using Cyp3a11$^{-/-}$ mice in association with vitamin E, which could therefore represent a starting point for future investigations. Unfortunately, Cyp4f14/Cyp3a11 double knockout mice – an animal model with a likely completely eliminated vitamin E metabolism – are not commercially available because they are probably not viable. Beside the use of knockout models, co-administration of vitamin E and sesamin, *i.e.* an inhibitor of Cyp4f14/Cyp3a11 (= CYP4F2/CYP3A4) dependent ω-hydroxylation (Sontag und Parker 2002), represents another approach for the investigation of vitamin E metabolism in rodent models. This method was successfully applied by Pein *et al.* to investigate α-T-13'-COOH, α-T-11'-COOH and α-T-9'-COOH concentrations in murine plasma and exudates (Pein et al. 2018). Based on their promising results, the use of sesamin could also be considered for future *in vivo* studies on vitamin E metabolism.

8.3.2.2 Tissue distribution

UPLC-MS/MS based analysis of vitamin E-derived LCM concentrations in blood, exudates and cells from α-TOH fed mice was approved as a reliable approach for the investigation of LCM distribution in *vivo* (Pein et al. 2018). In addition, Raman spectroscopic analysis together with the administration of deuterated LCMs or deuterated forms of their vitamin precursors could represents another promising tool for the identification of LCM distribution patterns in various tissues (Stiebing et al. 2017). Unfortunately, the use of deuterated compounds is expensive and therefore likely not practicable for large animal studies.

8.3.2.3 Pharmacokinetics

To elucidate pharmacokinetics of LCM metabolism, rodents could be treated with defined doses of deuterated vitamin E to enable the selective determination of newly formed metabolites (Traber et al. 2017). Afterwards, blood and tissues samples could be withdrawn from the animal at different time points to determine LCM concentrations in blood and tissues. The generated data will provide useful information on suitable dosages for future *in vivo* experiments as well as enable to estimate how fast the LCMs arrive at their potential sites of action.

8.3.2.4 LCM stability

Information about the stability against xenobiotic degradation are of special interest for *in vivo* studies that includes direct administration of the LCMs. The study of Pein *et al.* provided

first evidence for a minor degradation of α-T-13'-COOH in mice after intraperitoneal injection of the compound. Hence, α-T-13'-COOH concentration in plasma and exudates remained on a constant level after its initial increase due to compound injection. In contrast, δ-TE-13'-COOH concentrations in plasma and exudates did not increase after intraperitoneal injection of the compound, indicating that δ-TE-13'-COOH is immediately degraded by xenobiotic metabolism (Pein et al. 2018). This finding is further strengthened by the results of manuscript IV, where weekly injection of δ-TE-13-COOH had no influence on inflammatory processes in atherosclerotic mice, potentially because of the rapid degradation of the compound (Wallert et al. 2019). To deepen the knowledge on this issue, comparable studies should be carried out for all LCM forms. In addition, chemically modified LCMs, *i.e.* inaccessible for degradation via β-oxidation due to the replacement of single hydrogen atoms by fluorine atoms (suggested by Prof. Dr. Marc Birringer), could be used as a further reference for these experiments.

8.3.2.5 Identification of physiological relevant forms of vitamin E LCM

The investigation of Pein *et al.* showed that α-T-13'-COOH selectively accumulated in peritoneal leukocytes of mice that where fed α-TOH enriched diets. In contrast, other α-LCMs, such as α-T-11'-COOH and α-T-9'-COOH, were not detected (Pein et al. 2018). On the one hand, these results provide further evidence for the physiological significance of the vitamin E-derived LCMs, at least for α-T-13'-COOH, but on the other hand they raise a new question whether there are differences between the physiological relevance of the different LCM forms. To address this question, comparable *in vivo* studies based on a-, β-, γ-, and δ-TOH as well as a-, β-, γ-, and δ-TE enriched diets should be carried out, using an extended analytical spectrum of potential target tissues and cells. Especially the comparison of the physiologically relevance of α-TOH metabolites (considered the most important form of vitamin E in humans (Azzi 2019)) and γ-TOH metabolites (most relevant form for human nutrition in the USA (Péter et al. 2019)) could be of interest.

Beside their use for the elucidation of physiological fundamentals, animal models could also be used for a more targeted characterization of disease relevant functions and applications of the LCMs, as it was already done for atherosclerosis and immune function (Wallert et al. 2019; Pein et al. 2018).

8.3.3 Human studies

From my personal point of view, the implementation of investigations on physiologic fundamentals of vitamin E-derived LCMs in humans will be difficult in the nearby future. As already explained, these kind of investigations should initially be limited to animal models for ethical reasons (extraction of tissue samples, administration of deuterated compounds, inhibition of metabolic pathways), reasons of inter-individual variation (higher variation within humans compared with cultured laboratory animals) and handling reasons. Although the transferability of data from animal models to humans is not completely possible, the findings in animals can reveal first insights into LCM physiology that can be expanded by smaller studies on selected aspects of human LCM physiology, using tissue samples from biopsies or surgeries. However, the next decade of LCM research in humans should rather be focused on

the systematic collection and investigation of blood samples. To maximize the knowledge outcome from this easy accessible study material, LCM concentrations and distribution patterns should be analyzed in blood samples of healthy volunteers and patients with various types of non-communicable diseases. The collected data would potentially provide useful information on particularities of LCM concentrations or distribution ratios among their different forms in non-communicable diseases. This seems to be even more important, since recent studies indicated potential health benefits of vitamin E supplementation in cardiovascular diseases (Wiener et al. 2019), diabetes (Hodis und Mack 2019), metabolic syndrome (Bruno 2019), nonalcoholic fatty liver disease (NAFLD) (Banini und Sanyal 2019), asthmatic disease (Cook-Mills 2019) and Alzheimer's disease (Eckert 2019). Thus, the collected data could be used for specific applications, such as personalized vitamin E therapies. The potential use of personalized vitamin E administration was already indicated by large-scale human trails at the beginning of the 21th century. In general, these studies failed to provide clear evidence for health-promoting effects of vitamin E supplementation in the secondary prevention of cardiovascular diseases in a broad study population (Wiener et al. 2019). Interestingly, health promoting effects of vitamin E were only limited to certain parts of the overall study population (Lodge 2019). The reason for these individual differences were later identified in the pioglitazone or vitamin E for nonalcoholic steatohepatitis (PIVENS) trial. In this study, supplementation with 800 IU *all-rac*-α-TOH acetate for 96 days improved the hepatic histology of non-alcoholic steatohepatitis patients. However, not all patients of the α-TOH intervention group showed the same grade of histological improvement. Thus, Cheng *et al.* used fifteen subjects that either responded or did not respond to vitamin E supplementation (based on a decrease in NAFLD score) for additional metabolomics profiling. Indeed, a number of metabolites could be classified as biomarkers of vitamin E response (Cheng et al. 2012). Unfortunately, the analysis of Cheng *et al.* as well as further studies on biomarkers for vitamin E responders and non-responders did not consider the vitamin E-derived LCMs (Da Costa et al. 2013; West et al. 2015; Cole et al. 2013). Hence, current metabolomics approaches should be expanded by the determination of vitamin E-derived LCMs to prove their suitability as new potential biomarkers of the vitamin E effect on NAFLD. If the concept holds true and LCMs, such as α-T-13'-COOH, represent the biologically active forms of vitamin E as well as reliable biomarkers for the vitamin E effect on NAFLD in humans, determination of vitamin E-derived LCMs in blood could help to identify suitable patients for personalized and cost-efficient vitamin E therapies (Lodge 2019; Nuijten 2019).

9 Summary

With only two years away from the 100th anniversary of the discovery of vitamin E as an essential nutrient in 1922, we have still more questions than answers concerning its significance for human health. During the past hundred years, vitamin E research was characterized by great hopes but also bitter disappointments, which finally led to a strong decline of interest in this research area. However, vitamin E research is currently experiencing a scientific 'renaissance', which is among others attributed to the unrevealing of vitamin E metabolism in humans and to the elucidation of biological functions of the newly formed metabolites. Since their initial determination in human serum, especially the long-chain metabolites (LCMs), *i.e.* the first metabolites formed during the hepatic degradation of vitamin E, appeared as a class of molecules with putative relevance in the human body. Driven by a small community of ambitious scientist, research on these compounds has made great progress over the last decade, leading to the establishment of the LCMs as an acknowledged area of vitamin E research.

As one of the founding members of the LCM community, the work of our group is predominantly focused on the advance of this new and promising field of vitamin E research. Hence, the ten manuscripts included in this thesis also contribute to the progress of key areas in the field of LCM research, *i.e.* elucidation of biological functions, identification of signaling pathways and the comparison of LCM functionality with their vitamin precursors. The main contributions of the included research articles to the progress of LCM research can be summarized as follows:

(i) Discovery of hitherto unknown functions of the LCMs in inflammation and cellular lipid metabolism, *i.e.* inhibition of nitric oxide and prostaglandin formation, modulation of cellular lipid uptake and storage as well as prevention of VLDL-mediated foam cell formation.

(ii) Development of a new methodical approach for the investigation of LCM-mediated effects on lipoprotein lipase activity.

(iii) First description of a causal connection between gene regulatory effects of α-tocopherol-derived 13'-carboxychromanol (α-T-13'-COOH) and the peroxisome proliferator-activated receptor δ.

(iv) Description of differences concerning potency and function of α-tocopherol and α-T-13'-COOH on two hitherto unknown aspects of the cellular lipid homeostasis, *i.e.* regulation of PLIN2 expression and ANGPTL4-dependent regulation of LPL activity.

(v) Generation of indications for an atheroprotective potential of α-T-13'-COOH.

Despite of this great progress on fundamental areas of LCM functionality, their exact modes of action as well as their significance for human health are still a puzzle waiting to be solved. Especially the lack of data on LCM physiology in a living organism represent a great limitation of current and the major challenge of future investigations. Nevertheless, the already existing

studies on vitamin E-derived LCMs (including the manuscripts I to X) enabled the generation of major working hypotheses for future LCM research:

(i) The LCMs have a physiological relevance for human physiology and health.

(ii) The LCMs represent functional and therefore active forms of their vitamin precursors.

(iii) Essentiality of vitamin E for human health is closely linked to the LCMs.

However, further studies are required to finally confirm these hypotheses.

10 Zusammenfassung

Zwei Jahre vor dem 100. Jahrestag der Entdeckung von Vitamin E als essentiellen Nährstoff im Jahr 1922 gibt es immer noch viele ungeklärte Fragen hinsichtlich der Bedeutung des Vitamins für die menschliche Gesundheit. Die Vitamin-E-Forschung der letzten 100 Jahre war diesbezüglich von großen Hoffnung, aber auch herben Enttäuschung geprägt, die letztlich zu einem starken Interessenrückgang an diesem Forschungsbereich geführt haben. Derzeit erlebt die Vitamin-E-Forschung allerdings eine Renaissance, was unter anderem auf die Entdeckung des Metabolismus von Vitamin E sowie auf die Aufklärung von biologischen Funktionen der neu gebildeten Metaboliten zurückzuführen ist. Seit dem initialen Nachweis der langkettigen Metaboliten von Vitamin E (LCMs) (den ersten Metaboliten die während des Abbaus von Vitamin E in der Leber gebildet werden) im humanen Serum wird dieser Stoffklasse eine vermeintliche Relevanz für den menschlichen Körper nachgesagt. Angetrieben von einer kleinen Gemeinschaft ambitionierter Wissenschaftler hat die Forschung an diesen Verbindungen in den letzten zehn Jahren große Fortschritte erzielt und zur Etablierung der LCMs als anerkanntes Gebiet innerhalb der Vitamin-E-Forschung geführt.

Als eines der Gründungsmitglieder der „LCM Community“ konzentriert sich die Arbeit unserer Gruppe vorwiegend auf das Vorantreiben dieses neuen und vielversprechenden Forschungsbereiches. Auch die zehn in dieser Arbeit enthaltenen Manuskripte tragen dabei wesentlich zum wissenschaftlichen Fortschritt in Schlüsselbereichen auf dem Gebiet der LCM-Forschung bei. Diese umfassen unter anderem die Aufklärung biologischer Funktionen, die Identifizierung von Signalwegen und den funktionellen Vergleich der LCMs mit ihren Vitaminvorstufen. Die genauen Beiträge der in dieser Dissertation enthaltenen Forschungsartikel zum wissenschaftlichen Fortschritt auf dem Gebiet der LCM-Forschung lassen sich wie folgt zusammenfassen:

(i) Die Entdeckung bisher unbekannter Funktionen der LCMs bei Entzündungsprozessen und innerhalb des zellulären Lipidstoffwechsels, d.h. die Hemmung der Stickstoffmonoxid- und Prostaglandin Produktion, die Modulation der zellulären Lipidaufnahme und Lipidspeicherung, sowie einer Schutzfunktion vor VLDL-vermittelter Schaumzellbildung.

(ii) Die Entwicklung eines neuen methodischen Ansatzes zur Untersuchung von LCM-vermittelten Effekten auf die Lipoproteinlipase-Aktivität.

(iii) Die erste Beschreibung eines kausalen Zusammenhangs zwischen der genregulatorischen Wirkung von α-Tocopherol-abgeleitetem-α-13'-Carboxychromanol (α-T-13'-COOH) und dem Peroxisom-Proliferator-aktivierten Rezeptor δ.

(iv) Beschreibung von Unterschieden hinsichtlich Wirksamkeit und Funktion von α-Tocopherol und α-T-13'-COOH in Bezug auf zwei bisher unbekannte Aspekte der zellulären Lipidhomöostase, d.h. der Regulation der Expression von Adipophilin und der Angiopoietin-like 4 abhängigen Regulation der Aktivität der Lipoproteinlipase.

(v) Die Generierung von Hinweisen auf ein atheroprotektives Potential von α-T-13'-COOH.

Trotz dieser großen Fortschritte in grundlegenden Bereichen der LCM-Funktionalität sind ihre genauen Wirkmechanismen sowie ihre Bedeutung für die menschliche Gesundheit immer noch unklar. Insbesondere die fehlenden Daten zur Physiologie der LCMs im lebenden Organismus stellen einen großen Mangel aktueller und die größte Herausforderung für zukünftige Untersuchungen dar. Jedoch ermöglichen die bereits vorhandenen Studien zu den aus Vitamin E gebildeten LCMs (einschließlich der Manuskripte I bis X) die Generierung von wichtigen Arbeitshypothesen für die zukünftige Forschung auf diesem Gebiet:

(i) Die LCMs sind von physiologischer Relevanz für den menschlichen Körper.

(ii) Die LCMs stellen funktionelle und daher aktive Formen ihrer Vitaminvorläufer dar.

(iii) Die Relevanz von Vitamin E für die menschliche Gesundheit ist eng mit den LCMs verbunden.

Für die endgültige Bestätigung dieser Hypothesen sind jedoch weitere Untersuchungen erforderlich.

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B Eigenständigkeitserklärung

Hiermit erkläre ich an Eidesstatt, dass:

(i) mir die geltende Promotionsordnung der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist.

(ii) ich die vorliegende Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe und alle benutzen Hilfsmittel, persönliche Mitteilungen und Quellen in der Arbeit angegeben habe.

(iii) mich lediglich die in den Manuskripten angegebenen Personen bei der Auswahl und der Auswertung des Materials sowie bei der Erstellung der Manuskripte unterstützt haben.

(iv) ich nicht die Hilfe eines Promotionsvermittlers in Anspruch genommen habe.

(v) Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

(vi) ich diese Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe.

(vii) ich weder die gleiche oder eine in wesentlichen Teilen ähnliche, noch eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, März 2021 _____

Stefan Kluge

C Publications

Original Publications:

Kluge, S.; Boermel, L; Schubert, M.; Lorkowski, S. The vitamin E long-chain metabolite α-13'-COOH affects macrophage foam cell formation via modulation of the lipoprotein lipase system. *BBA - Molecular and Cell Biology of Lipids* **2021**; 1866:158875

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E Supplement